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Publication date:
2016

Document Version
Publisher's PDF, also known as Version of record

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Het verschijnen van dit proefschrift werd mede mogelijk gemaakt met steun van de Stichting Peribosch, de Jeroen Bosch Academie en Tranzo (Tilburg School of Social and Behavioral Sciences van de Universiteit van Tilburg).

ISBN: 978-94-6295-540-0
Layout and Design: www.wenz iD.nl || wendy schoneveld
Printed by: Uitgeverij BOXpress || proefschriftmaken.nl

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What can we learn from disease classifications and patient registries?

Proefschrift ter verkrijging van de graad van doctor
aan Tilburg University
op gezag van de rector magnificus,
prof. dr. E.H.L. Aarts,
in het openbaar te verdedigen ten overstaan van een
door het college voor promoties aangewezen commissie
in de aula van de Universiteit
op woensdag 14 december 2016
om 16:00 uur

door

Ellen Johanna Hubertina Schatorjé

geboren op 27 december 1983 te Venlo

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INTRODUCTION

Childhood is the period of growth and development. This is also true for the immune system, which contains all elements at birth but requires further development and education to reach full potential. Initially, defense against invading microorganisms relies mostly on innate immunity and protection by transplacentally acquired maternal immunoglobulins; the child's own adaptive immunity develops gradually to a fully expanded and matured immune system towards adulthood. Defects anywhere in this process can lead to immunodeficiency causing difficulties in the handling of infections. The immunodeficiency is called primary when these defects are determined by factors in the host. The immunodeficiency is called secondary when external influences like human immunodeficiency virus or side effects of medication are the cause. This thesis focuses on primary immunodeficiency. A wide variety of primary immunodeficiencies (PIDs) have been described, with predominantly antibody deficiencies (PADs) being the most common [1].

This introduction discusses the general principles of immunity, focusing on the adaptive immune system including the normal development of B- and T-lymphocytes, followed by the characteristics of PADs with a special emphasis on the hypogammaglobulinemias including the current classification schemes and use of patient registries. Finally, the aims of this thesis are outlined.

General principles of immunity

The immune system, which protects us against infections, consists of two major elements: *innate* and *adaptive* immunity. The innate immune system is a highly conserved response that provides immediate host defense. Next to physical and chemical barriers, the innate immune system comprises circulating effector proteins and cells with innate phagocytic activity: neutrophils, macrophages and natural killer (NK) cells. These immune cells respond quickly, but sometimes damage normal tissues due to lack of specificity. The adaptive immune system can generate an antigen specific reaction of B- and T-lymphocytes and can lead to the generation of immunological memory [2].

The adaptive immune system

The adaptive immune system is a tightly regulated network of B-lymphocytes ('humoral' immunity) and T-lymphocytes ('cellular' immunity); it connects with the innate immune system through antigen presenting cells (APCs; originating from the innate as well as adaptive systems). After developing and selection in the primary lymphoid organs (thymus and bone marrow), lymphocytes travel to the secondary lymphoid organs (lymph nodes and spleen) where they become activated by their encounter with antigens. During early development, each lymphocyte creates a unique antigen receptor resulting in a large and diverse repertoire of antigen specific receptors with the goal to be able to recognize all potential pathogens. When a lymphocyte recognizes an antigen for the first time, this

leads to (1) clonal proliferation with production of large numbers of effector cells and (2) production of long-lived memory cells which can be quickly activated in the future when the same antigen is encountered again [3].

Normal B cell development

B cells develop from hematopoietic stem cells in the bone marrow, where they pass through several developmental stages without encountering antigen. Here, they create their antigen specificity embedded in their unique B cell receptor (BCR). The BCR possesses an antigen binding moiety which is composed of a membrane-bound antibody that has a unique and randomly determined antigen-binding site. Initially the BCR membrane-bound immunoglobulin molecule is of the IgM isotype. Furthermore, the BCR contains a signal transduction moiety: a heterodimer called CD79, consisting of Ig-α and Ig-β. Both Ig-α/Ig-β span the plasma membrane and have a cytoplasmic tail bearing an immunoreceptor tyrosine-based activation motif (ITAM) which can induce intra-cellular signal transduction after binding of tyrosine kinases. The uniqueness of each B cell receptor is created by so-called V(D)J recombination in the immunoglobulin genes. The immunoglobulin genes are made up of three different types of segments: V (variable), D (diversity) and J (joining). During the rearrangement process, one V, one D and one J segment are randomly joined together to form a heavy chain (IgH). Later, one V and one J segment are randomly combined to form a light chain. During this VJ rearrangement of the light chain, fragments of DNA are separated from the BCR; these DNA strains remain in a stable episomal form in the nucleus, known as kappa-deleting recombination excision circles (KRECs) [4]. Every BCR eventually consists of two identical heavy chains and either two Igκ or Igλ light chains . These light chains consist of a constant (C_l) and a variable (V_l) domain. The kappa and lambda light chains have no apparent functional differences. The heavy chains determine the different immunoglobulin isotypes: α - IgA 1, 2, δ – IgD, γ - IgG 1, 2, 3, 4, ε - IgE, μ - IgM [3, 5]. Also the heavy chains consist of a constant (C_h) and a variable (V_h) region. The common hypervariable region of both chains is called the complementary determining region (CDRs). The variable (Fab) region of the immunoglobulin can bind to antigens, whereas the constant (Fc) domain receptor is a ligand for Fc receptors on innate immunity phagocytes. Different immunoglobulin isotypes differ in Fc fragment and therefore differ in Fc receptor affinity and specificity. If the process of creating a unique BCR finally leads to a functional BCR and the cell is not autoreactive, the cell migrates out of the bone marrow to the periphery as a transitional B cell. Transitional B cells are characterized by expression of bone marrow B cell markers like CD10, CD24, CD38 and CD44 [6].

Once these transitional B cells enter the peripheral blood they develop into naive (mature) B cells [7]. These naive B cells circulate through the lymphatic system and lymph nodes where they can encounter antigens. When the BCR of a naive B cell recognizes an antigen, another stimulus is necessary to activate the B cell into proliferation and differentiation. Most antigens (protein antigens) require participation of T cells (T-dependent antigens). Then, CD40 on the B cell and CD40L on the follicular helper

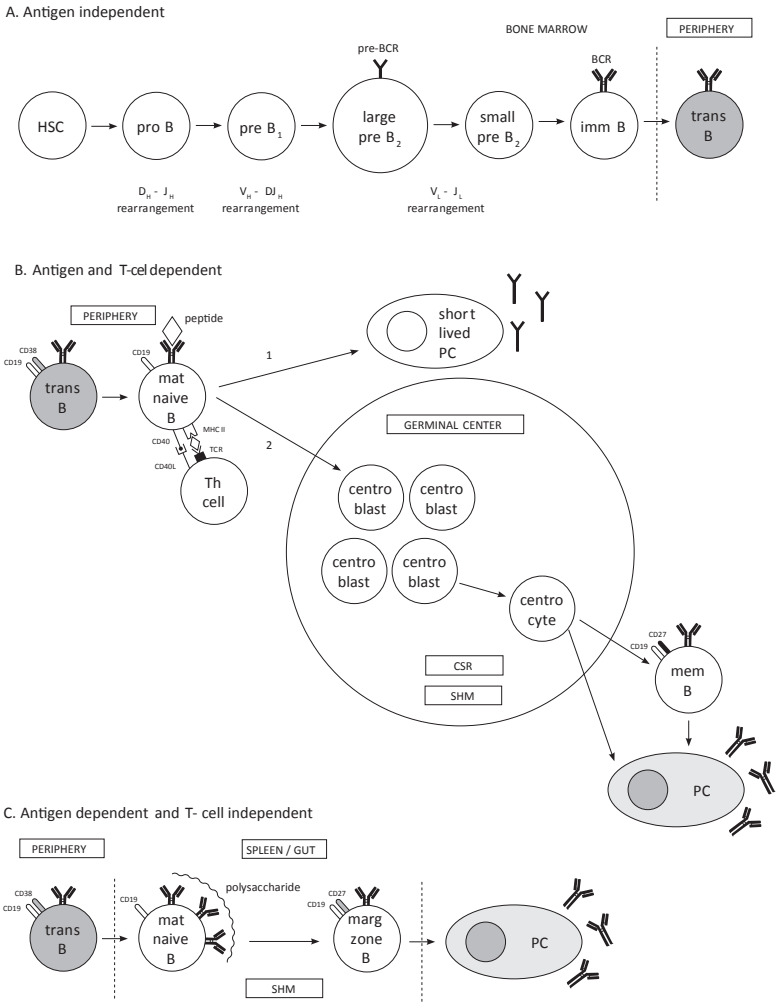

Figure 1. B cell development

A. antigen independent B cell development in the bone marrow.

BCR: B cell receptor, HSC: hematopoietic stem cell, imm B: immature B cell, trans B: transitional B cell

B. antigen and T cell dependent B cell development

1: differentiation of transitional B cell into short-lived plasma cell secreting low affinity antibodies without somatic hypermutation

2: proliferation and differentiation in germinal centers of lymph nodes leading to class-switched memory B cells or antibody secreting plasma cells

CD: cluster of differentiation, CSR: class switch recombination, mat naive B: mature naive B cell, mem B: memory B cell, MHCII: major histocompatibility complex type II, PC: plasma cell, SHM: somatic hypermutation, TCR: T cell receptor, Th cell: T helper cell, trans B: transitional B cell

CD19: B-lymphocyte antigen CD19, CD27: tumor necrosis factor receptor superfamily 7 (TNFRSF7), CD38: cyclic adenosine diphosphate (ADP) ribose hydrolase, CD40: tumor necrosis factor receptor superfamily 5 (TNFRSF5), CD40L: CD40 ligand, =CD154

C. antigen dependent and T cell independent B cell development occurring in the marginal zone of the spleen or lamina propria in the gut

Marg zone B: marginal zone B cell, mat naive B: mature naive B cell, PC: plasma cell, SHM: somatic hypermutation, trans B: transitional B cell

Adapted from Bonilla 2010, Le Bien 2008, Thesis Driessen 2013.

T cell interact at the margin between primary follicles and T cell areas in secondary lymphoid tissues. This, in combination with co-stimulatory signals from receptors such as ICOS (inducible T cell costimulator), CTLA-4 (cytotoxic T-lymphocyte-associated 4), CD28 and PD-1 (programmed cell death 1) as well as soluble factors such as interleukin (IL)-4 and IL-21, serves as the needed secondary stimulus [8]. After this, activated B cells will secrete immunoglobulins with the same specificity as their BCR. Activated B cells can enter one of two pathways. Either the B cell immediately becomes a short-lived plasma cell secreting low-affinity antibody without somatic hypermutation, or it enters a follicle to form a germinal center. In the germinal center the B cell can increase its affinity for antigens by induction of somatic hypermutations (SHM) in the variable regions of its Ig genes [9]. Furthermore, the B cell can switch from the production of IgM and IgD to one of the other isotypes, IgG, IgA, or IgE. This process is called class-switch recombination (CSR) [10]. Both processes, SHM and also CSR, are triggered by activation-induced cytidine deaminase (AID) [11]. The 'upgraded' B cell with high affinity BCR can differentiate into a memory B cell, which persists after antigen challenge and can rapidly expand during secondary responses, or into an antibody secreting plasma cell [12]. The Ig-isotypes differ in size, structure and function (Table 1).

Table 1. Properties of immunoglobulin isotypes/subclasses.

	Serum (%)	Structure	Function
IgG	75	Monomer	All (except IgG-4): Secondary immune response *(proteins: IgG1++, IgG2+, IgG3++)* *(polysaccharides: IgG1+, IgG2+++, IgG3+/-)* Complement fixation + Opsonisation +++ Neutralisation of toxins and viruses
IgG-1	67 of IgG	Monomer	
IgG-2	22 of IgG	Monomer	
IgG-3	7 of IgG	Monomer	
IgG-4	4 of IgG	Monomer	
IgM	10	Pentamer	Primary immune response Complement fixation +++ Opsonisation +
IgA	15	Monomer, dimer	Mucosal response
IgA-1		Monomer, dimer	
IgA-2		Monomer, dimer	
IgD	<0.5	Monomer	Unclear
IgE	<0.01	Monomer	Allergy Immunity against helminths

Ig = immunoglobulin. *Edited from Schroeder 2010 [18].*

T cell independent (TI) antigens (e.g. polysaccharide antigens) can activate B cells without the help of T cells. They can either activate via the BCR and other receptors such as toll-like receptors (TI-1) or via extensive cross-linking of the BCR because of the repetitive nature of the antigen, like pneumococcal polysaccharides (TI-2). This activation occurs in the marginal zone of the spleen or in the lamina propria in the gut [13, 14] and leads

to a memory B cell type with a high affinity BCR through SHM but without CSR [15]. Additional activation through the transmembrane activator and calcium-modulator and cyclophilin ligand interactor (TACI) can lead to class switch recombination and subsequent isotype switching in a pathway independent of CD40 [16]. However, the magnitude of isotype switching is substantially smaller than that induced by CD40 ligation [17].
The T cell independent B cell is called the marginal zone B cell or natural effector B cell. A summary of the surface markers of these peripheral B cell subpopulations is shown in Table 2.

Table 2. Surface markers of peripheral B cell subpopulations.

Cell type	Surface markers
Transitional B cell	$CD19^{+}CD38^{++}IgM^{++}$
Naive B cell	$CD19^{+}CD27^{-}IgM^{+}IgD^{+}$
Class switched plasmablast	$CD19^{+}CD38^{+++}IgM^{-}$
Natural effector B cell	$CD19^{+}CD27^{+}IgM^{+}IgD^{+}$
Switched memory B cell	$CD19^{+}CD27^{+}IgM^{-}IgD^{-}$

CD=cluster of differentiation. Ig=immunoglobulin. *Adapted from Bonilla 2010 [3].*

Normal T cell development

T cells develop from common lymphoid progenitors originating from the bone marrow or – in the fetus – liver [19]. Under the influence of IL-7, these cells develop into T cell progenitors in the thymus. Further differentiation of the progenitor cells is characterized by coordinated series of genomic rearrangements creating functional genes encoding the α and β or γ and δ chains of the T cell antigen receptor (TCR). Then VDJ rearrangement similar to the process described in B cell development occurs, creating a wide diversity of different TCRs. In this process of VDJ rearrangement fragments of DNA are excised from the TCR; these DNA strains remain in a stable episomal form in the nucleus, known as T cell receptor excision circles (TRECs) [3].
Surface expression of an αβ or γδ TCR marks the transition from a pre-T cell to a double positive thymocyte (expressing CD4 as well as CD8 on their surface), situated in the outer cortex of the thymus. Further differentiation into single positive thymocytes is found in the medulla of the thymus. This is regulated by a process of positive and negative selection involving major histocompatibility complex (MHC) molecules. Thymocytes that interact with epithelial MHC class I molecules will retain their CD8, whereas those selected on MHC class II retain their CD4, both becoming single-positive T-lymphocytes. However, negative selection is exerted on T cells of which the TCR binds with very high avidity to self-MHC molecules, ensuring that auto-reactive T cell precursors are not permitted to mature. These fully differentiated but antigen-naive cells leave the thymus by entering the circulation [3].

In the periphery, T cells are activated by interaction of their TCR with antigenic peptide - MHC complexes on APCs [20]. This activation can lead to several different effector cell types.

The largest subgroup of T cells in the blood is the $CD4^+$ $TCR\alpha\beta^+$ population. These cells coordinate the cells and interactions in the network and are therefore called T helper (Th) cells. They originate from the antigen-naive $CD4^+$ cells (Th0). Different Th cell subtypes have been recognized; Th1 and Th2 cells are the major players as effector T cells [3].

- Th1 cells: these cells differentiate from naive Th0 cells under the influence of IL-12 and produce cytokines (interferon [IFN]-γ and IL-2) that stimulate cell-mediated responses activating mononuclear phagocytes, NK cells, and cytolytic T cells into killing of intracellular microbes and virally infected targets [3].
- Th2 cells: stimulated by IL-4 Th0 cells differentiate into the second form of helper cells producing different cytokines (IL-4, IL-5, IL-10 and IL-13) enhancing antigen production and also hypersensitivity, and parasite induced immune responses.
- Th17 cells: they are named after their secretion of IL-17, a potent pro-inflammatory cytokine. Different types of IL-17 are capable of driving granulocyte recruitment and tissue damage; they have a role in auto-inflammatory and chronic allergic responses [21].
- Treg cells: these $CD25^+$ regulatory T cells also express CD4 and can dampen the immune activation [22].
- Tfh cells: this special subgroup of Th cells in lymph nodes and spleen is called follicular Th cells. These $CD4^+$ memory T cells express the chemokine receptor CXCR5, triggering B cells and leading to GC formation [3].

Cytotoxic T cells expressing the CD8 molecule can destroy cells by binding to their MHC class I molecules, which present peptides from intracellular pathogens or transformed proteins. This leads to production of granzymes and perforin activating apoptosis of the infected cell. Simultaneous binding of Fas ligand on Fas (CD95) of the target cells further triggers this apoptotic process. The $CD8^+$ cells represent a major fraction of the circulating T cells [3].

Only a small subset of T cells express γδ TCR. Most of these T cells are double negative (no CD4 or CD8). Possibly, they have a role in the response to mycobacterial antigens.

Helper T cells as well as cytotoxic T cells can differentiate into memory cells. This differentiation is influenced by the duration and intensity of antigenic stimulation [23]. A summary of the surface markers of these peripheral T cell subpopulations is shown in Table 3.

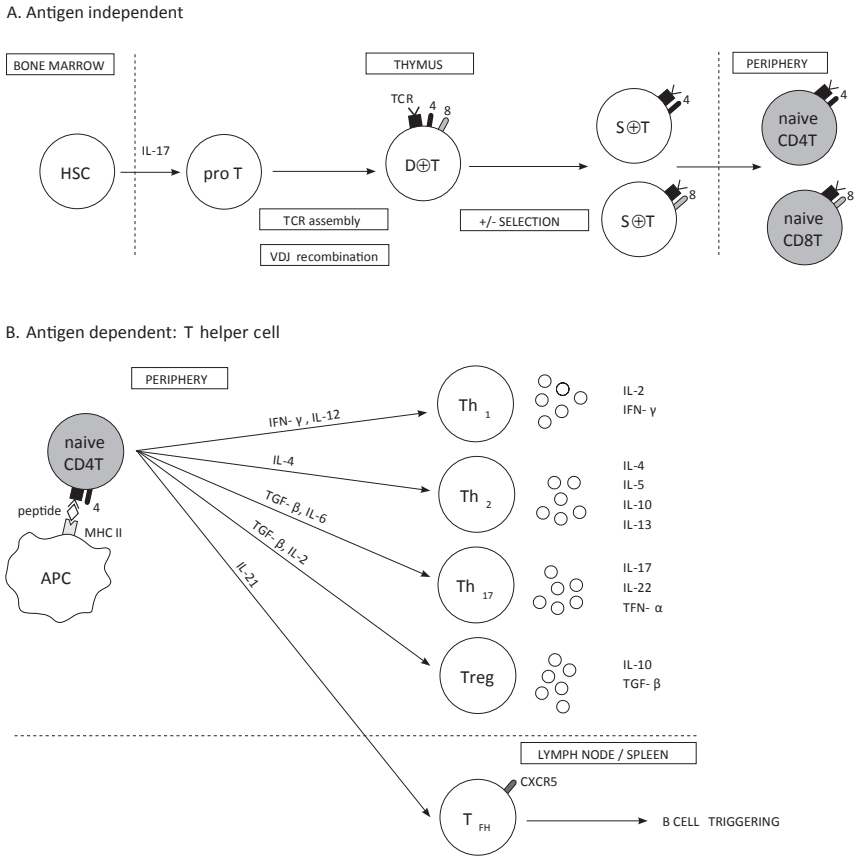

Figure 2. T cell development

A. antigen independent T cell development in the thymus. *D+T: double positive T cell, HSC: hematopoietic stem cell, S+T: single positive T cell, TCR: T cell receptor. 4: CD4, 8: CD8.*

B. antigen dependent development of T helper cell in the peripheral blood and lymph node/spleen into different effector cells. *APC: antigen presenting cell, CXCR5: chemokine receptor 5, MHC: major histocompatibility complex, Tc: cytotoxic cell*

C. antigen dependent development of cytotoxic T cell. *MHC: major histocompatibility complex, Tc: cytotoxic T cell*
Adapted from Bonilla 2010

Table 3. Surface markers of peripheral T cell subpopulations.

Cell type	Surface markers
Naive T cell	$CD45RA^{+}CD27^{+}CD28^{+}CCR7^{+}$
Terminally differentiated T cell	$CD45RA^{+}CD27^{-}CD28^{-}CCR7^{-}$
Effector memory T cell	$CD45RA^{-}CD27^{-}CD28^{-}CCR7^{-}$
Central memory T cell	$CD45RA^{-}CD27^{+}CD28^{+}CCR7^{+}$

Adapted from Saule 2006 [24].

The developing immune system in children

Prenatally, the immune system is in favor of immune suppression and even in the first weeks after birth the immune responses are still mostly suppressive. The innate immune system relies on very ancient responses such as the production of superoxides. Further innate immune maturation takes place in the next years, but full capacity is not reached until teenage [25].

In the adaptive immune system, there is a massive increase in lymphocytes in the first weeks after birth. This is largely independent of gestational age at birth [26]. This expansion applies for B cells as well as T cells. They further increase during the first years of life, and then normalize to adult levels in school children [27]. Numbers of memory B cells are low at birth, this is explained by the lack of antigenic stimulation during prenatal life [26].

Transplacental transport of IgG during the third trimester protects infants during the first six months of life. In newborns IgM is the most commonly produced immunoglobulin. During the first years of life more class switching occurs and at one year of age IgG levels have reached 70% of adult levels but IgA levels are still only 30% of adult levels [25].

Young children have an impaired antibody response to polysaccharide antigens. This T cell independent response to encapsulated bacteria occurs mainly in the marginal zone of the spleen, which is not fully developed until two years of age [25].

Predominantly antibody deficiencies

Predominantly antibody deficiencies (PADs) result from developmental defects in the B cell population. The hallmark is a marked reduction or absence of immunoglobulins, with an increased susceptibility to mostly bacterial infections that typically involve the upper and lower respiratory tract. No exact incidence of PAD is known. An epidemiological study in a single state in the USA showed an incidence of 98:100.000 persons [28]. Several genetic defects underlying PADs have been described [29]. Because of the limited genotype-phenotype correlation additional genetic and/or environmental factors probably play a role [30].

Defects in the early stages of B cell development (mostly defects in the pre-BCR molecule or pre-BCR signaling pathway) can lead to the absence of circulating mature B cells and

all immunoglobulin isotypes. The most common cause of an early B cell defect is X-linked agammaglobulinemia caused by a mutation in the gene for Bruton's kinase (BTK) [31]. Other defects in the pre-BCR can lead to rare, autosomal-recessive forms of agammaglobulinaemia: defects in the μ-heavy chain (IGHM) [32] or the λ5 chain [33], the pre-BCR and BCR co-receptors Igα and Igβ (CD79α and CD79β) [34, 35] and components of the pre-BCR and BCR signaling pathways, including the p85α subunit of phosphoinositide 3-kinase (PI3K) [36] and the scaffold protein B cell linker (BLNK) [37]. Defects in CSR also lead to very low levels of IgG, IgA and IgE, but accompanied by normal to increased IgM levels. Depending on the molecular defect, SHM can be affected as well [30].
B cell defects in later phases of the developmental pathway can lead to various forms of hypogammaglobulinemia. This group of PADs is by far the most common entity of PIDs, comprising nearly half of all diagnoses in the European Society for Immunodeficiencies (ESID) online database [1]; the molecular basis of these diseases is as yet largely unknown.

The clinical picture of hypogammaglobulinemia
Patients with hypogammaglobulinemia suffer from recurrent ear-nose-throat (ENT) and airway infections. Hypogammaglobulinemias comprise a heterogeneous group of diseases in which at least one of the immunoglobulin isotypes, subtypes, or functional antibody types is decreased.

Common variable immunodeficiency disorders (CVID) is the most severe form with an estimated prevalence of 1:25,000. Most patients are detected in (young) adulthood, but symptoms often start during childhood, and more than one third of CVID diagnoses are made in children <14 years of age [38]. CVID is defined by a marked decrease of IgG and marked decrease of IgA with or without low IgM (at least 2 SD below the mean for age), with impaired response to immunization and/or low switched memory B cells (<70% of age-related normal value). The diagnosis should not be made before 4 years of age. Other defined causes of hypogammaglobulinemia as well as profound T cell deficiency have to be excluded before a diagnosis of CVID can be made (www.esid.org; probable CVID; accessed on April 25, 2016). Furthermore, CVID is associated with an increased susceptibility to autoimmune disorders and malignancies [39, 40]. Patients with idiopathic primary hypogammaglobulinemia who do not fulfill the CVID criteria show similar infectious episodes, but lack the autoimmune complications [41]. Several gene defects have been described in CVID in the past decade: inducible costimulatory (ICOS) [42], TACI [43], B cell activation factor receptor (BAFF-R) [44], lipopolysaccharide responsive beige-like anchor protein (LRBA) [45], phospholipase Cγ2 (PLCG2) [46], protein C kinase δ (PRKCD) [47], CD19 [48], CD20 [49], CD21 [50],CD81 [51] and NF-κB1 Subunit p50 [52]. However, these gene defects are responsible for only 5-10% of the CVID patients; the genetic background in the majority of patients is still unknown. Several CVID classification schemes based on immunophenotyping of B cells have been published. Low numbers

of switched memory B cells are associated with splenomegaly, granulomas [53, 54] and lymphoproliferation [53], whereas high numbers of transitional B cells in patients with low switched memory B cells can increase the risk of lymphadenopathy [54]. High numbers of immature $CD21^{-/low}$ B cells are associated with increased incidence of splenomegaly [54, 55] and autoimmune cytopenia [55]. Division of CVID patients into clinical phenotypes showed that only the group with polyclonal lymphocytic infiltration had a 5-fold increased risk of lymphoid malignancy. Predictive markers for this polyclonal lymphocytic infiltration were higher levels of IgM and lower circulating CD8 cells [56].
Other forms of hypogammaglobulinemia can exist as isolated phenomenon or appear in combination. Especially the combination of more than one partial antibody deficiency will lead to clinically significant disease with recurrent respiratory tract infections [57]. Nothing is as yet known about the genetic background of these forms of hypogammaglobulinemia, with the exception of increased susceptibility to *IgA-deficiency* in case of a TACI mutation [58]. Patients with IgA-deficiency can suffer from autoimmunity and allergy in addition to the recurrent infectious episodes. On the other hand, 85-90% of IgA-deficient people are asymptomatic [59].

IgG-subclass deficiency is defined as a deficiency in one or more IgG-subclasses (>2 SD below age-matched reference values) with normal or near normal IgG concentration. *Specific polysaccharide antibody deficiency (SPAD)* is diagnosed when there is profound alteration of the antibody response to polysaccharide antigens, either after documented invasive infection with e.g. S. pneumoniae or after test immunization with an unconjugated pneumococcal (or other) polysaccharide vaccine. In both diseases, T cell and more severe B cell defects should be excluded (www.esid.org; accessed on April 25, 2016). Patients with IgG-subclass deficiency or SPAD can be asymptomatic, but if not typically present with increased susceptibility to bacterial ENT and respiratory tract infections. Of the IgG-subclass deficiencies especially IgG2 deficiency seems to be relevant, the more so when combined with SPAD [60].

Hypogammaglobulinemia in children

Hypogammaglobulinemia is the most common form of immunodeficiency in children, but the exact prevalence is not known [61]. It is important to note that every child starts with a 'physiological hypogammaglobulinemia' during the first six months of life, the immunoglobulin levels increase during normal immune maturation. In some cases this hypogammaglobulinemia persists longer, leading to so-called *transient hypogammaglobulinemia of infancy (THI)*. THI generally resolves before the age of 2 years [62], but may last much longer [63]. The diagnosis can only be made with certainty in retrospect. If the hypogammaglobulinemia does not resolve with age, nor fits the diagnostic criteria of CVID, it is named 'unclassified'. Children with 'unclassified' hypogammaglobulinemia suffer from a higher frequency of infectious episodes and autoimmune disease compared to children with THI [64]. At the moment it is not possible to predict which child will develop a more or less severe immunodeficiency in the future, and which child will recover.

Impaired response to polysaccharide antigens is a physiological phenomenon in children below the age of 2 years, reflecting the ongoing B cell development during the first years of life. However, the majority of infants show an adequate response to certain pneumococcal serotypes [65]. From 2 to 3 years of age onwards, children can mount an adequate response to polysaccharide antibodies; in some cases this physiological non-responsiveness can last until around 6 years of age. Because most polysaccharide antibodies are found in the IgG_2 subclass, IgG_2 subclass deficiency is often associated with SPAD [61].

Hypogammaglobulinemia and chromosomal disorders

Several PID syndromes have been identified and increasingly their genetic background has been unraveled [66]. Syndromes with chromosomal abnormalities of number or structure are considered as a distinct group of PID syndromes [67]. Well-known examples are Down syndrome (trisomy 21) which is associated with T and B cell abnormalities [68] and DiGeorge syndrome (22q11 deletion) which is associated with mild to very severe T cell dysfunction [69]. Also, Turner syndrome [70] and Wolf-Hirschhorn syndrome [71] are known to be associated with immunodeficiency, mostly antibody disorders. It is as yet unknown how many other chromosomal aberrations are associated with immunological abnormalities.

Diagnostic challenge of PAD in children

Identifying children with PAD, especially the milder forms like IgG-subclass deficiency and/or SPAD, among the many children seen in everyday practice can be challenging. In healthy children aged 0-2 years up to 11 respiratory infections per year, up to 8 infections per year for children 3-5 years and up to 4 infections per year for children 6-12 years is considered normal [72]. At least 6% of the children presents to a physician with recurrent respiratory infections [73, 74]. Most of these are self-limiting, viral upper respiratory tract infections. The infections are typically seasonal, with a higher incidence in autumn and winter when children are exposed to a large number of viruses at home and in day-care centres or nursery schools. These infections are generally mild and do not lead to hospitalization. In general between infectious episodes children are well and show normal growth [75].

So, frequent upper respiratory tract infections in the first years of life are not exceptional, and this makes it difficult to decide which children with recurrent infections are at the end of the normal spectrum and which children need further immunological work-up. Studies show that the incidence of underlying PID in children with recurrent lower respiratory infections varies between 9.7%-16.1% [76-80]. The Jeffrey Model Foundation developed 10 warning signs for PID based on expert opinion to help physicians identifying patients at risk for PID [21]. Of these warning signs only a positive family history, parental consanguinity and the need for intravenous antibiotics are truly predictive for PAD in

retrospective studies among patients referred to tertiary centres [81, 82]. A limitation of these warning signs is that they focus mainly on the infections. Therefore, patients primarily presenting with other signs such as severe allergy, autoimmunity or malignancy may be missed [83].
Another challenge is formed by a group of children who spontaneously recover during follow-up, since hypogammaglobulinemia in young children can be a physiological phenomenon due to the developing immune system. Therefore, several diagnoses should not be made until a certain age. According to the ESID criteria, CVID and IgA-deficiency can only be diagnosed in children >4 years of age (www.esid.org). No age limits for the diagnosis of IgG-subclass deficiency and SPAD have been defined by ESID so far, but it is better not to use this diagnosis before the age of 3-4 years either.

Use of age-matched reference values for lymphocyte subpopulations

Immunophenotyping of lymphocyte subpopulations is an important tool in the diagnosis of immunological and hematological diseases. When absolute numbers of lymphocyte subpopulations fall outside predetermined reference ranges, this indicates possible disease. When this thesis was started, the available reference values comprised only a small selection of lymphocyte subpopulations, determined in relatively small groups; also, methods were used which are not used in daily (diagnostic) practice [27, 84-90]. Lymphocyte subpopulations were also increasingly used to classify patients with CVID into subgroups with different clinical prognosis according to the composition of their B-lymphocyte compartment [53-55]. These classifications were mainly developed with data obtained in adults, which does not mean these cutoffs are applicable in children.

The use of registries and surveys to study rare diseases

Because PIDs are relatively rare disorders, international collaboration is necessary to study the clinical characteristics of these diseases. Registries and surveys can be very useful tools in this process. Since 2004, the ESID has been running an online database for primary immunodeficiencies: the ESID Online Database. This database registers demographical, clinical and laboratory characteristics of patients with PID [91]. The aim of the ESID Online Database is to build a common data pool and estimate the disease burden of PID in Europe and to provide an internet-based database for clinical and research data on patients with PID. In 2014 the database contained 8002 patients <20 years of age (47% of total) (www.esid.org). In the Netherlands, the national surveillance institute for pediatric diseases ("Nederlands Signalerings Centrum voor Kindergeneeskunde" [NSCK]) collects data on specific diagnoses among all practicing pediatricians; from March 1, 2009, to November 1, 2011 data on children diagnosed with hypogammaglobulinemia were collected. Within ESID, researchers also collect data by sending around surveys among its members around a specific diagnosis or clinical research question.

Aim and outline of this thesis

The aims of the studies described in this thesis are:

1) to get more insight into the pitfalls the developing immune system creates for doctors confronted by children with potential primary immunodeficiency by:
   a. developing reliable age matched reference values for newly described lymphocyte subpopulations in children used in current PID diagnosis
   b. testing existing disease classification schemes for applicability in children
   c. studying the effect of age on somatic hypermutation levels in children
2) to study in detail the clinical picture and characteristics of children with antibody deficiencies focusing on hypogammaglobulinemia using data from (inter)national registries and surveys.

In *Part 1* the age-related reference values of different players of the immune system in healthy children are shown. *Chapter 1* presents the reference values of B cell subpopulations in children in comparison with adult values, and discusses the impact of the obtained results on the use of current adult classification schemes for CVID in children. *Chapter 2* focuses on the peripheral T cell compartment of healthy children. In *chapter 3* the levels of somatic hypermutations in B cell receptors in healthy children using the IgK-restriction enzyme hot-spot mutation assay (IgK-REHMA) are shown.
Part 2 shows the clinical characteristics of pediatric hypogammaglobulinemia in different study populations. In *chapter 4* a large European cohort of children with hypogammaglobulinemia is described using data of the ESID Online Database. *Chapter 5* discusses the challenge of IgG-subclass deficiency and specific polysaccharide antibody deficiency in a Dutch pediatric cohort obtained through the system of the Dutch Pediatric Surveillance Unit. A cohort of patients with chromosomal aberrations and primary immunodeficiency, consisting mainly of antibody deficiency, collected through an international ESID survey is presented in *chapter 6*.

Literature

1. Gathmann B, Grimbacher B, Beaute J, Dudoit Y, Mahlaoui N, Fischer A, et al. The European internet-based patient and research database for primary immunodeficiencies: results 2006-2008. Clin Exp Immunol. 2009;157 Suppl 1:3-11.
2. Parkin J, Cohen B. An overview of the immune system. Lancet. 2001;357(9270):1777-89.
3. Bonilla FA, Oettgen HC. Adaptive immunity. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S33-40.
4. van Zelm MC, Szczepanski T, van der Burg M, van Dongen JJ. Replication history of B-lymphocytes reveals homeostatic proliferation and extensive antigen-induced B cell expansion. The Journal of experimental medicine. 2007;204(3):645-55.
5. van Zelm MC, van der Burg M, de Ridder D, Barendregt BH, de Haas EF, Reinders MJ, et al. Ig gene rearrangement steps are initiated in early human precursor B cell subsets and correlate with specific transcription factor expression. J Immunol. 2005;175(9):5912-22.
6. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood. 2005;105(11):4390-8.
7. Chung JB, Silverman M, Monroe JG. Transitional B cells: step by step towards immune competence. Trends Immunol. 2003;24(6):343-9.
8. Allen CD, Okada T, Cyster JG. Germinal-center organization and cellular dynamics. Immunity. 2007;27(2):190-202.
9. Teng G, Papavasiliou FN. Immunoglobulin somatic hypermutation. Annu Rev Genet. 2007;41:107-20.
10. Stavnezer J, Guikema JE, Schrader CE. Mechanism and regulation of class switch recombination. Annu Rev Immunol. 2008;26:261-92.
11. Muramatsu M, Kinoshita K, Fagarasan S, Yamada S, Shinkai Y, Honjo T. Class switch recombination and hypermutation require activation-induced cytidine deaminase (AID), a potential RNA editing enzyme. Cell. 2000;102(5):553-63.
12. LeBien TW, Tedder TF. B-lymphocytes: how they develop and function. Blood. 2008;112(5):1570-80.
13. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood. 2011;118(8):2150-8.
14. Mond JJ, Vos Q, Lees A, Snapper CM. T cell independent antigens. Curr Opin Immunol. 1995;7(3):349-54.
15. Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood. 2004;104(12):3647-54.
16. Castigli E, Wilson SA, Garibyan L, Rachid R, Bonilla F, Schneider L, et al. TACI is mutant in common variable immunodeficiency and IgA deficiency. Nat Genet. 2005;37(8):829-34.
17. Lee JJ, Ozcan E, Rauter I, Geha RS. Transmembrane activator and calcium-modulator and cyclophilin ligand interactor mutations in common variable immunodeficiency. Curr Opin Allergy Clin Immunol. 2008;8(6):520-6.
18. Schroeder HW, Jr., Cavacini L. Structure and function of immunoglobulins. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S41-52.
19. Takahama Y. Journey through the thymus: stromal guides for T cell development and selection. Nat Rev Immunol. 2006;6(2):127-35.
20. Dustin ML. The cellular context of T cell signaling. Immunity. 2009;30(4):482-92.
21. Steinman L. A brief history of T(H)17, the first major revision in the T(H)1/T(H)2 hypothesis of T cell-mediated tissue damage. Nat Med. 2007;13(2):139-45.
22. Chatila TA. Role of regulatory T cells in human diseases. J Allergy Clin Immunol. 2005;116(5):949-59; quiz 60.
23. Sallusto F, Geginat J, Lanzavecchia A. Central memory and effector memory T cell subsets: function, generation, and maintenance. Annu Rev Immunol. 2004;22:745-63.
24. Saule P, Trauet J, Dutriez V, Lekeux V, Dessaint JP, Labalette M. Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment. Mech Ageing Dev. 2006;127(3):274-81.

25. Ygberg S, Nilsson A. The developing immune system - from foetus to toddler. Acta Paediatr. 2012;101(2):120-7.
26. Walker JC, Smolders MA, Gemen EF, Antonius TA, Leuvenink J, de Vries E. Development of lymphocyte subpopulations in preterm infants. Scand J Immunol. 2011;73(1):53-8.
27. Comans-Bitter WM, de Groot R, van den Beemd R, Neijens HJ, Hop WC, Groeneveld K, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr. 1997;130(3):388-93.
28. Joshi AY, Iyer VN, Hagan JB, St Sauver JL, Boyce TG. Incidence and temporal trends of primary immunodeficiency: a population-based cohort study. Mayo Clin Proc. 2009;84(1):16-22.
29. van der Burg M, van Zelm MC, Driessen GJ, van Dongen JJ. New frontiers of primary antibody deficiencies. Cell Mol Life Sci. 2012;69(1):59-73.
30. Durandy A, Kracker S, Fischer A. Primary antibody deficiencies. Nat Rev Immunol. 2013;13(7):519-33.
31. Vetrie D, Vorechovsky I, Sideras P, Holland J, Davies A, Flinter F, et al. The gene involved in X-linked agammaglobulinaemia is a member of the src family of protein-tyrosine kinases. Nature. 1993;361(6409):226-33.
32. Yel L, Minegishi Y, Coustan-Smith E, Buckley RH, Trubel H, Pachman LM, et al. Mutations in the mu heavy-chain gene in patients with agammaglobulinemia. N Engl J Med. 1996;335(20):1486-93.
33. Minegishi Y, Coustan-Smith E, Wang YH, Cooper MD, Campana D, Conley ME. Mutations in the human lambda5/14.1 gene result in B cell deficiency and agammaglobulinemia. The Journal of experimental medicine. 1998;187(1):71-7.
34. Minegishi Y, Coustan-Smith E, Rapalus L, Ersoy F, Campana D, Conley ME. Mutations in Igalpha (CD79a) result in a complete block in B cell development. J Clin Invest. 1999;104(8):1115-21.
35. Dobbs AK, Yang T, Farmer D, Kager L, Parolini O, Conley ME. Cutting edge: a hypomorphic mutation in Igbeta (CD79b) in a patient with immunodeficiency and a leaky defect in B cell development. J Immunol. 2007;179(4):2055-9.
36. Conley ME, Dobbs AK, Quintana AM, Bosompem A, Wang YD, Coustan-Smith E, et al. Agammaglobulinemia and absent B lineage cells in a patient lacking the p85alpha subunit of PI3K. The Journal of experimental medicine. 2012;209(3):463-70.
37. Minegishi Y, Rohrer J, Coustan-Smith E, Lederman HM, Pappu R, Campana D, et al. An essential role for BLNK in human B cell development. Science. 1999;286(5446):1954-7.
38. Quinti I, Soresina A, Spadaro G, Martino S, Donnanno S, Agostini C, et al. Long-term follow-up and outcome of a large cohort of patients with common variable immunodeficiency. J Clin Immunol. 2007;27(3):308-16.
39. Haymore BR, Mikita CP, Tsokos GC. Common variable immune deficiency (CVID) presenting as an autoimmune disease: role of memory B cells. Autoimmun Rev. 2008;7(4):309-12.
40. Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol. 1999;92(1):34-48.
41. Driessen GJ, Dalm VA, van Hagen PM, Grashoff HA, Hartwig NG, van Rossum AM, et al. Common variable immunodeficiency and idiopathic primary hypogammaglobulinemia: two different conditions within the same disease spectrum. Haematologica. 2013;98(10):1617-23.
42. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, et al. Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol. 2003;4(3):261-8.
43. Salzer U, Chapel HM, Webster AD, Pan-Hammarstrom Q, Schmitt-Graeff A, Schlesier M, et al. Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. Nat Genet. 2005;37(8):820-8.
44. Warnatz K, Salzer U, Rizzi M, Fischer B, Gutenberger S, Bohm J, et al. B cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. Proc Natl Acad Sci U S A. 2009;106(33):13945-50.
45. Lopez-Herrera G, Tampella G, Pan-Hammarstrom Q, Herholz P, Trujillo-Vargas CM, Phadwal K, et al. Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. Am J Hum Genet. 2012;90(6):986-1001.

46. Zhou Q, Lee GS, Brady J, Datta S, Katan M, Sheikh A, et al. A hypermorphic missense mutation in PLCG2, encoding phospholipase Cgamma2, causes a dominantly inherited autoinflammatory disease with immunodeficiency. Am J Hum Genet. 2012;91(4):713-20.
47. Salzer E, Santos-Valente E, Klaver S, Ban SA, Emminger W, Prengemann NK, et al. B cell deficiency and severe autoimmunity caused by deficiency of protein kinase C delta. Blood. 2013;121(16):3112-6.
48. van Zelm MC, Reisli I, van der Burg M, Castano D, van Noesel CJ, van Tol MJ, et al. An antibody-deficiency syndrome due to mutations in the CD19 gene. N Engl J Med. 2006;354(18):1901-12.
49. Kuijpers TW, Bende RJ, Baars PA, Grummels A, Derks IA, Dolman KM, et al. CD20 deficiency in humans results in impaired T cell-independent antibody responses. J Clin Invest. 2010;120(1):214-22.
50. Thiel J, Kimmig L, Salzer U, Grudzien M, Lebrecht D, Hagena T, et al. Genetic CD21 deficiency is associated with hypogammaglobulinemia. J Allergy Clin Immunol. 2012;129(3):801-10 e6.
51. van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, et al. CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest. 2010;120(4):1265-74.
52. Fliegauf M, Bryant VL, Frede N, Slade C, Woon ST, Lehnert K, et al. Haploinsufficiency of the NF-kappaB1 Subunit p50 in Common Variable Immunodeficiency. Am J Hum Genet. 2015;97(3):389-403.
53. Piqueras B, Lavenu-Bombled C, Galicier L, Bergeron-van der Cruyssen F, Mouthon L, Chevret S, et al. Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. J Clin Immunol. 2003;23(5):385-400.
54. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood. 2008;111(1):77-85.
55. Warnatz K, Denz A, Drager R, Braun M, Groth C, Wolff-Vorbeck G, et al. Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood. 2002;99(5):1544-51.
56. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood. 2008;112(2):277-86.
57. Aghamohammadi A, Cheraghi T, Gharagozlou M, Movahedi M, Rezaei N, Yeganeh M, et al. IgA deficiency: correlation between clinical and immunological phenotypes. J Clin Immunol. 2009;29(1):130-6.
58. Rachid R, Castigli E, Geha RS, Bonilla FA. TACI mutation in common variable immunodeficiency and IgA deficiency. Curr Allergy Asthma Rep. 2006;6(5):357-62.
59. Yel L. Selective IgA deficiency. J Clin Immunol. 2010;30(1):10-6.
60. Cheng YK, Decker PA, O'Byrne MM, Weiler CR. Clinical and laboratory characteristics of 75 patients with specific polysaccharide antibody deficiency syndrome. Ann Allergy Asthma Immunol. 2006;97(3):306-11.
61. Stiehm ER. The four most common pediatric immunodeficiencies. J Immunotoxicol. 2008;5(2):227-34.
62. Notarangelo LD. Primary immunodeficiencies. J Allergy Clin Immunol. 2010;125(2 Suppl 2):S182-94.
63. Keles S, Artac H, Kara R, Gokturk B, Ozen A, Reisli I. Transient hypogammaglobulinemia and unclassified hypogammaglobulinemia: 'similarities and differences'. Pediatr Allergy Immunol. 2010;21(5):843-51.
64. Moschese V, Graziani S, Avanzini MA, Carsetti R, Marconi M, La Rocca M, et al. A prospective study on children with initial diagnosis of transient hypogammaglobulinemia of infancy: results from the Italian Primary Immunodeficiency Network. Int J Immunopathol Pharmacol. 2008;21(2):343-52.
65. Balloch A, Licciardi PV, Russell FM, Mulholland EK, Tang ML. Infants aged 12 months can mount adequate serotype-specific IgG responses to pneumococcal polysaccharide vaccine. J Allergy Clin Immunol. 2010;126(2):395-7.
66. Bousfiha AA, Jeddane L, Ailal F, Al Herz W, Conley ME, Cunningham-Rundles C, et al. A phenotypic approach for IUIS PID classification and diagnosis: guidelines for clinicians at the bedside. J Clin Immunol. 2013;33(6):1078-87.
67. Ming JE, Stiehm ER, Graham JM, Jr. Syndromic immunodeficiencies: genetic syndromes associated with immune abnormalities. Crit Rev Clin Lab Sci. 2003;40(6):587-642.
68. Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol. 2009;156(2):189-93.

69. Davies EG. Immunodeficiency in DiGeorge Syndrome and Options for Treating Cases with Complete Athymia. Front Immunol. 2013;4:322.
70. Lorini R, Ugazio AG, Cammareri V, Larizza D, Castellazzi AM, Brugo MA, et al. Immunoglobulin levels, T cell markers, mitogen responsiveness and thymic hormone activity in Turner's syndrome. Thymus. 1983;5(2):61-6.
71. Hanley-Lopez J, Estabrooks LL, Stiehm R. Antibody deficiency in Wolf-Hirschhorn syndrome. J Pediatr. 1998;133(1):141-3.
72. Gruber C, Keil T, Kulig M, Roll S, Wahn U, Wahn V, et al. History of respiratory infections in the first 12 yr among children from a birth cohort. Pediatr Allergy Immunol. 2008;19(6):505-12.
73. de Martino M, Ballotti S. The child with recurrent respiratory infections: normal or not? Pediatr Allergy Immunol. 2007;18 Suppl 18:13-8.
74. Ruskamp JM, Hoekstra MO, Postma DS, Kerkhof M, Bottema RW, Koppelman GH, et al. Exploring the role of polymorphisms in ficolin genes in respiratory tract infections in children. Clin Exp Immunol. 2009;155(3):433-40.
75. Patria MF, Esposito S. Recurrent lower respiratory tract infections in children: a practical approach to diagnosis. Paediatr Respir Rev. 2013;14(1):53-60.
76. Cabezuelo Huerta G, Vidal Mico S, Abeledo Gomez A, Frontera Izquierdo P. [Underlying causes of recurrent pneumonia]. An Pediatr (Barc). 2005;63(5):409-12.
77. Ciftci E, Gunes M, Koksal Y, Ince E, Dogru U. Underlying causes of recurrent pneumonia in Turkish children in a university hospital. J Trop Pediatr. 2003;49(4):212-5.
78. Hoving MF, Brand PL. Causes of recurrent pneumonia in children in a general hospital. J Paediatr Child Health. 2013;49(3):E208-12.
79. Lodha R, Puranik M, Natchu UC, Kabra SK. Recurrent pneumonia in children: clinical profile and underlying causes. Acta Paediatr. 2002;91(11):1170-3.
80. Owayed AF, Campbell DM, Wang EE. Underlying causes of recurrent pneumonia in children. Arch Pediatr Adolesc Med. 2000;154(2):190-4.
81. Reda SM, El-Ghoneimy DH, Afifi HM. Clinical predictors of primary immunodeficiency diseases in children. Allergy Asthma Immunol Res. 2013;5(2):88-95.
82. Subbarayan A, Colarusso G, Hughes SM, Gennery AR, Slatter M, Cant AJ, et al. Clinical features that identify children with primary immunodeficiency diseases. Pediatrics. 2011;127(5):810-6.
83. de Vries E, European Society for Immunodeficiencies m. Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol. 2012;167(1):108-19.
84. Piatosa B, Wolska-Kusnierz B, Pac M, Siewiera K, Galkowska E, Bernatowska E. B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. Cytometry Part B, Clinical cytometry. 2010;78(6):372-81.
85. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Hop WC, Boerma GJ, et al. Longitudinal survey of lymphocyte subpopulations in the first year of life. Pediatr Res. 2000;47(4 Pt 1):528-37.
86. Erkeller-Yuksel FM, Deneys V, Yuksel B, Hannet I, Hulstaert F, Hamilton C, et al. Age-related changes in human blood lymphocyte subpopulations. J Pediatr. 1992;120(2 Pt 1):216-22.
87. Huck K, Feyen O, Ghosh S, Beltz K, Bellert S, Niehues T. Memory B cells in healthy and antibody-deficient children. Clin Immunol. 2009;131(1):50-9.
88. Morbach H, Eichhorn EM, Liese JG, Girschick HJ. Reference values for B cell subpopulations from infancy to adulthood. Clin Exp Immunol. 2010;162(2):271-9.

89. Shearer WT, Rosenblatt HM, Gelman RS, Oyomopito R, Plaeger S, Stiehm ER, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol. 2003;112(5):973-80.
90. van Gent R, van Tilburg CM, Nibbelke EE, Otto SA, Gaiser JF, Janssens-Korpela PL, et al. Refined characterization and reference values of the pediatric T- and B cell compartments. Clin Immunol. 2009;133(1):95-107.
91. Guzman D, Veit D, Knerr V, Kindle G, Gathmann B, Eades-Perner AM, et al. The ESID Online Database network. Bioinformatics. 2007;23(5):654-5.

PART ONE

Peripheral Lymphocyte Development during Childhood

Determination of Reliable Age Matched Reference Values for Newly Described Lymphocyte Subpopulations in Children

CHAPTER 1

Age-matched Reference Values for B-Lymphocyte Subpopulations and CVID Classifications in Children

Scand J Immunol. 2011 Nov;74(5):502-10.

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Abstract

Age-matched reference values are generally presented with 5th and 95th percentiles as 'normal' reference range. However, they are mostly determined in relatively small groups, which renders this presentation inaccurate. We determined reference values for B-lymphocyte subpopulations in healthy children with the statistical method of tolerance intervals that deals far better with the relatively small numbers tested, and compared these to the cut-off values used in the currently used EUROclass classification for common variable immunodeficiency disorders (CVID) in children. CVID is a heterogeneous group of primary immunodeficiency diseases characterized by low serum immunoglobulin levels and inadequate response to vaccination. Disease-modifying heterozygous amino acid substitutions in TACI are found in around ±10% of CVID patients. Interestingly, we found that age is the primary determinant of TACI-expression on B-lymphocytes, independent of switched memory B-lymphocyte numbers. Immunophenotyping of B-lymphocyte subpopulations is increasingly used to classify patients with CVID into subgroups with different clinical prognosis according to the composition of their B-lymphocyte compartment. These classifications were mainly developed with data obtained in adults. Because of the maturing paediatric immune system, they may not be equally applicable in children: our and other age-matched reference values show great changes in the composition of the B-lymphocyte compartment during development. Although the greatest changes in B-lymphocyte subpopulations occur below the age of 2 years, when the diagnosis of CVID cannot yet be made, it is likely that a classification developed in adults cannot be used to classify the prognosis of children.

Introduction

Common variable immunodeficiency disorders (CVID) is a heterogeneous group of primary immunodeficiency diseases characterized by late-onset hypogammaglobulinaemia [1]. The diagnosis is based on low serum immunoglobulin levels, an inadequate response to vaccination, and exclusion of other causes of hypogammaglobulinaemia [1]. The diagnosis should not be made before the age of 2–4 years [2]. It is more difficult to make an accurate diagnosis of CVID in children than in adults, because other primary immunodeficiency diseases like X-linked agammaglobulinaemia may not have been detected yet in young children. Also, CVID develops gradually: IgA deficiency, IgG-subclass deficiencies, IgM deficiency, anti-polysaccharide and/or anti-protein antibody deficiencies accumulate until full-blown hypogammaglobulinaemia is present [3]. With an estimated prevalence of 1:10,000 ranging to 1:50,000, CVID is the most common symptomatic primary antibody deficiency, not only in adults, but in children as well [1, 4]. The prevalence of CVID increases with age [5]. It can also be difficult to distinguish developing CVID from delayed maturation of the immune system in so-called transient hypogammaglobulinaemia, which is relatively common especially in younger children [6]. The majority of CVID patients present with recurrent bacterial infections of the respiratory tract. In some patients with CVID, ultimately T-lymphocyte function deteriorates as well [7]. Gastrointestinal disease, lymphoproliferative disorders, autoimmune phenomena, and granulomatous inflammation are seen in subgroups of patients; in some patients these precede the recurrent infections [8]. Up to 73% of CVID patients develop chronic structural pulmonary complications. Although the incidence is lower, these pulmonary abnormalities are already present in children with CVID [9, 10]. Patients are treated with life-long replacement of immunoglobulins, but even with adequate immunoglobulin substitution chronic lung disease will develop in the majority of patients [11]. The exact aetiology of CVID is unknown, but causative gene mutations have been reported in a few families, including CD19 [12], CD20, B cell activating factor receptor (BAFF-R), the inducible costimulator (ICOS), and CD80 genes [13] and around 10% of CVID patients show disease-modifying heterozygous amino acid substitutions in the transmembrane and calcium-modulating cyclophilin ligand (CAML) interactor (TACI) [13, 14].

Immunophenotyping of lymphocyte subpopulations is an important tool in the diagnosis of immunological and haematological diseases. When absolute numbers of lymphocyte subpopulations fall outside predetermined reference ranges, this indicates possible disease. Lymphocyte subpopulations are also increasingly used to classify patients with CVID into subgroups with different clinical prognosis according to the composition of their B-lymphocyte compartment [15–17]. These classifications were mainly developed with data obtained in adults, however. Because of their maturing immune system, these classifications may not be equally applicable in children: age-matched reference values that have been determined for B-lymphocyte subpopulations in children show great changes in the composition of the B-lymphocyte compartment during development [18–26]. Not only do the absolute number of $CD19^+$ B-lymphocytes show a massive

expansion shortly after birth, the relative distribution between naive ($CD19^+CD27^-IgD^+$), natural effector ($CD19^+CD27^+IgD^+$), switched memory ($CD19^+$ $CD27^+IgD^-$) [18, 20, 23, 24, 26], and $CD21^{low}$ ($CD19^+CD21^{low}CD38^{low}$) B-lymphocytes [24], as well as class-switched plasmablasts ($CD19^+CD38^{+++}IgM^-$) and transitional B cells ($CD19^+CD38^{++}IgM^{++}$) [18] also change significantly with increasing age. The most important shifts in B-lymphocyte subpopulations take place in the first weeks to months after birth, but development continues until adulthood.

Up to now, age-matched reference values are generally presented with 5th and 95th percentiles as 'normal' reference range. However, these studies were performed in relatively small groups, especially in the group(s) of youngest children, which renders this presentation inaccurate. For instance, in a group of 20 patients, the 5th percentile is determined only by the value obtained in the patient with rank order 2, and the 95th percentile only by the value obtained in the patient with rank order 19, implying that the distribution of the other sampled values does not play any role in inferring the percentile limits.

We therefore determined reference values for B-lymphocyte subpopulations in healthy children using the statistical method of tolerance intervals that deals far better with the relatively small numbers tested, and used them to evaluate the applicability of the currently used EUROclass classification for CVID to children.

Material and methods

Subjects and samples

Leftover ethylenediaminetetraaceticacid (EDTA) blood from healthy children, who underwent venipuncture or blood sampling by heel prick or finger prick for other reasons, was used for the study. We also asked parents of otherwise healthy infants visiting the paediatric outpatient clinic permission to perform a venipuncture, heel prick, or finger prick for study purposes only; after informed consent 1–2 ml of EDTA blood was taken. Neonatal cord blood was obtained by venipuncture immediately after clamping of the cord. Patients with an active infection, diseases of the immune system, or on immunosuppressive therapy were excluded. Below 2 years of age, patients with perinatal problems such as prematurity (gestational age <35 weeks), birth weight <p10 or >p90, congenital or perinatal infection, artificial delivery, congenital deformities and suspected metabolic or neurological disease were also excluded. The study population was divided into ten age groups according to Comans-Bitter et al. [22]: neonatal cord blood (group 1), 1 week to 2 months (group 2), 2–5 months (group 3), 5–9 months (group 4), 9–15 months (group 5), 15–24 months (group 6), 2–5 years (group 7), 5–10 years (group 8), 10–16 years (group 9), and 16 years and older (group 10). Blood samples were obtained between April 2008 and January 2011. This study was approved by the local Medical Ethics Committee.

Flowcytometric analysis

Four-color flowcytometric immunophenotyping with directly labelled monoclonal antibodies (MAb) was used to determine the following lymphocyte subpopulations:

T-lymphocytes ($CD3^+$), B-lymphocytes ($CD19^+$), natural killer (NK) cells ($CD3^+CD16^+$and/or $CD56^+$), naive B-lymphocytes ($CD19^+CD27^-IgM^+IgD^+$), natural effector B-lymphocytes ($CD19^+CD27^+IgM^+IgD^+$), IgM only memory B-lymphocytes ($CD19^+CD27^+IgM^+IgD^-$), switched memory B-lymphocytes ($CD19^+CD27^+IgM^-IgD^-$), transitional B cells ($CD19^+CD38^{++}IgM^{++}$), CD21low B cells ($CD19^+CD21^{low}CD38^{low}$), and class-switched plasmablasts ($CD19^+CD38^{+++}IgM^-$). We also analyzed the expression of CD5, CD10, CD20, CD24, CD38, Igκ and Igλ, and TACI and BAFF-R on $CD19^+$ B-lymphocytes.

Aliquots were incubated for 15 min in the dark at room temperature with a mixture of optimally titrated MAbs within 24 h after sampling. The antibodies we used are CD3 fluoresceïne-isothiocyanate (FITC), CD5 FITC, CD38 FITC, CD4 phycoerythrin (PE), CD16 PE, CD20 PE, CD24 PE, CD56 PE, BAFF-R PE, CD8 peridinin chlorophyll protein–cyanin (PerCP-Cy-5.5), CD19 PerCP-Cy5.5, CD45 PerCP-Cy5.5, CD10 allophycocyanin (APC), CD14 APC, CD21 APC, CD27 APC [all Becton Dickinson (BD), San Jose, California USA], SmIgκ FITC, SmIgD FITC, SmIgλ PE, SmIgM PE (Dakopatts, Glostrup, Denmark), CD235a FITC, CD71 PE (Sanquin, Amsterdam, The Netherlands) and TACI Biotin (Peprotech, Rocky Hill, USA)/streptavidine APC (BD). Before surface staining, erythrocytes were lysed with ammonium chloride (NH_4Cl). Remaining cells were washed twice with phosphate buffered saline/bovine serum albumin 0.5%, and analysed with a FACSCalibur flowcytometer (BD) using CellQuestPro software. Calibration of the flowcytometer took place with CaliBRITE beads according to the manufacturer's instructions (BD) en daily quality control with Cyto-Cal (microgenics Duke Scientific, Fremont CA, USA) following the guidelines of Kraan et al. [27]. The lymphogate was checked with a CD3/CD14 labelling and considered correct if less than 1% monocyte contamination was present. T-lymphocytes and NK-cells were used to check the 'lymphosum' (B+T+NK = 100 ± 5%).

Leukocyte count and differential were determined with a routine haematology analyzer (XE 2100, Sysmex, Kobe, Japan). In neonatal cord blood, the lymphogate was corrected for contamination with erythroid cells (normoblasts and unlysed erythrocytes) using the following formula: corrected % of lymphocyte subpopulation = % of lymphocyte subpopulation within the lymphogate x 100/[100 - (%$CD71^+$ normoblasts + %$CD235^+CD71^-$ unlysed erythrocytes within the lymphogate)]. The absolute size of each lymphocyte subpopulation was calculated by multiplying the relative size of the lymphocyte subpopulation and the absolute lymphocyte count.

Statistics

The number of subjects in the different age groups varied between 10 and 21 per tested subpopulation; numbers that are too low to determine robust percentile points at 5 and 95%. Confidence intervals may seem to offer an alternative, but deal with estimating the range of the population mean, and do not cover the distribution of the population values. The proper statistical procedure is to calculate the tolerance interval which enclosures a specific proportion of the population, estimated on the basis of the values sampled. The tolerance interval takes into account the sample size, the noise in the estimates of the mean and standard deviation, and the confidence about the tolerance interval [28].

We set the proportion to be included at 0.90 (two-sided, comparable to the percentile points p5 and p95), with a confidence level of 0.95.
Tolerance intervals assume normally distributed populations. Most of our data were positively skewed. In 75% of the cases the means were larger than the median (339 out of 450 subsets of measurements = 45 sets of measurements in 10 age groups). Logarithmic transformation reduced the number of cases to 42% (187), which is much closer to the expected percentage (50%). Two tests of normality were applied to each subset of the 450 measurements, the Kolmogorov–Smirnov test and the Shapiro–Wilks test. The original values returned 94 (21%) and 118 (26%) significant violations ($\alpha = 0.05$) of the normality assumption. The log-transformed values returned 33 (7%) and 42 (9%) violations, which is fairly close to the expected percentage (5%). The logarithmic transformation has the additional advantage that the estimated tolerance intervals do not include non-existing negative values. All values given in the tables are the re-transformed logarithmic values. To evaluate the age effect in the 45 sets of measurements a one-way ANOVA test ($\alpha = 0.05$) was applied.
The correlations of TACI and BAFF-R values with B cell subpopulations and age were assessed with the Pearson product-moment correlations and partial correlations. The logarithmic transformation was applied both to age in months (because of the large age range in the older groups; a value of 1 was added) and the measured values (because of their positive skewness). All calculations and tests were performed with SPSS 16.0 for Windows.

Results

B-lymphocyte subpopulations

Absolute B-lymphocyte numbers double during the first months of life and then gradually decrease almost fivefold from the second half of the first year of life to adult values; this is almost entirely caused by expansion of the naive B-lymphocyte pool, and to a small extent by expansion of transitional cells (Figure 1), which are higher in the youngest age groups.

Age-matched reference values

The absolute and relative sizes of the measured B-lymphocyte subpopulations are shown in Tables 1 and 2, respectively. The data were not normally distributed, given the means of the different subpopulations being larger than the median in 75% of the subsets of the measurements in the different age groups. We therefore used logarithmic values to calculate the value intervals (see 'Material and methods'). With the provided reference values in Tables 1 and 2, we give a 95% chance that 90% of healthy children will show absolute numbers within this range. All sets of measurements showed a statistically significant age effect ($\alpha = 0.05$), except for absolute and relative values of $CD19^{+}CD20^{-}$ B cells; this subpopulation was very small in number in all age-groups.

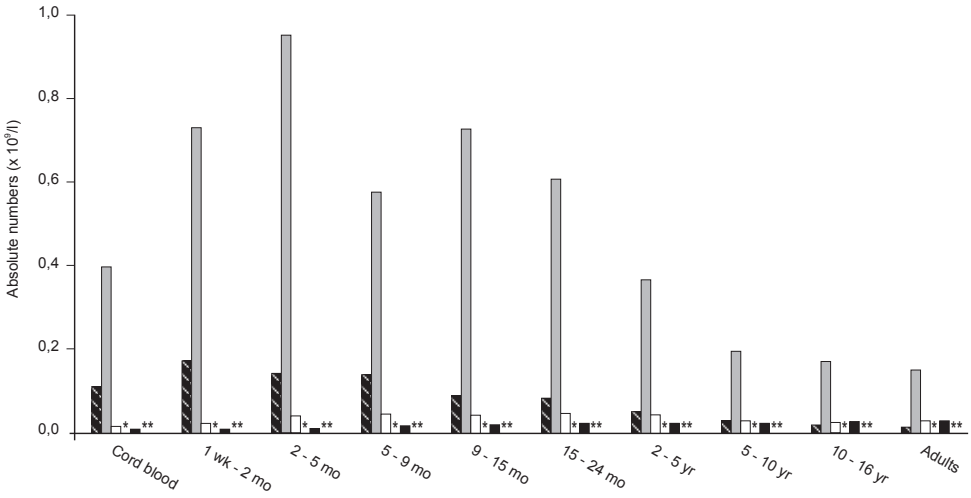

Figure 1
Composition of the B-lymphocyte compartment during childhood (median per age group). Diagonal striped bars transitional B cell ($CD19^+CD38^{++}IgM^{++}$). Grey bars naive B cell ($CD19^+CD27^-IgM^+IgD^+$). White bars natural effector B cell ($CD19^+CD27^+IgM^+IgD^+$). Asterisk IgM only memory B cell ($CD19^+CD27^+IgM^+IgD^-$). Black bars switched memory B cell ($CD19^+CD27^+IgM^-IgD^-$). Double asterisk class-switched plasmablast ($CD19^+CD38^{+++}IgM^-$).

TACI and BAFF-R expression

We determined TACI and BAFF-R expression in a randomly selected subgroup (total group n = 36; cord blood n = 6, 1 week to 2 months n = 2, 2–5 months n = 2, 5–9 months n = 3, 9–15 months n = 3, 15–24m n = 2, 2–5 years n = 2, 5–10 years n = 4, 10–16 years n = 4, adults n = 8). All children showed >95% BAFF-R positivity on $CD19^+$ cells, with a mean fluorescence intensity of 226 (on a scale of 1024 channels). The mean fluorescence intensity of TACI-expression was 11 (on a scale of 1024 channels) on TACI- $CD19^+$ cells (background staining) and 104 (on a scale of 1024 channels) on $TACI^+$ $CD19^+$ cells. A median of 2.6% (range 1.0–12.6%) of B-lymphocytes were TACI-positive.

No correlation was found between switched memory B-lymphocyte numbers and the percentage of $TACI^+$ B-lymphocytes ($r = 0.213$, $P = 0.213$); a negative correlation was found between naive B-lymphocyte numbers and the percentage of $TACI^+$ B-lymphocytes ($r = -0.738$, $P = 0.000$), and a positive correlation between the percentage of $TACI^+$ B-lymphocytes and age ($r = 0.538$, $P = 0.001$). A partial correlation was computed controlling for age to investigate whether the negative correlation between the percentage of $TACI^+$ B-lymphocytes and naive B-lymphocyte numbers was based on the developmental role of age only. After correction for age, the negative correlation between the percentage of $TACI^+$ B-lymphocytes and naive B-lymphocyte numbers disappeared ($r = -0.318$, $P = 0.063$), showing that age is the primary determinant of TACI-expression on B-lymphocytes.

Table 1 Absolute numbers of B-lymphocytes in peripheral blood.

Population	N =	Cord blood	N =	1 w – 2 m	N =	2 – 5 m	N =	5 – 9 m	N =	9 – 15 m	N =
Lymphocytes (total)	136	5.4 (3.1-9.4)	18	5.7 (2.9-11.4)	11	6.5 (3.4-12.2)	12	5.8 (1.8-18.7)	13	6.3 (3.2-12.3)	10
T-lymphocytes	136	3.1 (1.4-6.8)	18	4.0 (1.9-8.4)	11	4.5 (2.2-9.2)	12	4.0 (1.4-11.5)	13	4.4 (2.4-8.3)	10
NK-cells	136	1.2 (0.5-3.1)	18	0.51 (0.14-1.9)	11	0.44 (0.097-1.99)	12	0.52 (0.068-3.9)	13	0.50 (0.071-3.5)	10
B-lymphocytes	136	0.54 (0.14-2.0)	18	0.81 (0.18-3.5)	11	1.1 (0.52-2.3)	12	0.90 (0.13-6.3)	13	0.94 (0.11-7.7)	10
Transitional B cells	106	0.099 (0.015-0.65)	11	0.17 (0.023-1.2)	10	0.14 (0.053-0.37)	11	0.16 (0.015-1.7)	10	0.093 (0.016-0.53)	10
Naive B-lymphocytes	127	0.42 (0.097-1.8)	17	0.69 (0.14-3.5)	10	0.96 (0.46-2.0)	11	0.76 (0.11-5.3)	13	0.78 (0.097-6.4)	10
Natural effector B-lymphocytes	127	0.012 (0.0022-0.065)	17	0.021 (0.0042-0.11)	10	0.032 (0.0073-0.14)	11	0.032 (0.003-0.27)	13	0.035 (0.0024-0.52)	10
IgM-only B-lymphocytes	127	0.0004 (0.0000-0.0040)	17	0.0011 (0.0004-0.0026)	10	0.0025 (0.0003-0.021)	11	0.0038 (0.0005-0.029)	13	0.0038 (0.0005-0.046)	10
Switched memory B-lymphocytes	127	0.0033 (0.0008-0.013)	17	0.0040 (0.0008-0.020)	10	0.0083 (0.0011-0.060)	11	0.011 (0.0015-0.082)	13	0.015 (0.0018-0.13)	10
Class switched plasmablasts	106	0.0037 (0.0004-0.035)	11	0.0054 (0.0007-0.043)	10	0.0036 (0.0002-0.058)	11	0.0047 (0.0001-0.16)	10	0.0030 (0.0002-0.036)	10
$CD21^{low}/CD38^{low}$ B cells	108	0.013 (0.0023-0.074)	13	0.017 (0.0015-0.20)	10	0.027 (0.0086-0.082)	11	0.041 (0.0094-0.18)	10	0.025 (0.0029-0.23)	10
$CD24^{+}/CD38^{+}$ B cells	127	0.19 (0.052-0.73)	18	0.22 (0.033-1.4)	10	0.24 (0.065-0.86)	11	0.23 (0.042-1.27)	11	0.19 (0.025-1.5)	10
Ig-kappa	134	0.25 (0.065-0.99)	18	0.38 (0.072-2.0)	10	0.56 (0.26-1.2)	11	0.45 (0.057-3.5)	13	0.45 (0.050-4.1)	10
Ig-lambda	134	0.22 (0.053-0.93)	18	0.29 (0.056-1.6)	10	0.42 (0.20-0.90)	11	0.36 (0.057-2.2)	13	0.38 (0.039-3.6)	10
Kappa-lambda ratio	134	1.1 (0.79-1.6)	18	1.3 (0.77-2.1)	10	1.3 (0.96-1.8)	11	1.3 (0.85-1.9)	13	1.2 (0.75-1.9)	10
$CD20^{-}$ B cells	127	0.016 (0.0006-0.48)	18	0.0086 (0.0025-0.030)	10	0.0078 (0.0015-0.039)	11	0.011 (0.0015-0.084)	11	0.0087 (0.0021-0.036)	10
$CD5^{+}$ B cells	127	0.22 (0.031-1.6)	18	0.43 (0.051-3.6)	10	0.77 (0.39-1.6)	11	0.63 (0.076-5.2)	11	0.51 (0.65-4.0)	10
$CD10^{+}$ B cells	127	0.18 (0.035-0.90)	18	0.35 (0.062-2.0)	10	0.47 (0.19-1.2)	11	0.42 (0.073-2.4)	11	0.28 (0.052-1.5)	10

Mean and 90% range x $10^{e}9$/l. W = week, m = month, y = year.

Population	15 – 24 m	N =	2 -5 y	N =	5 – 10 y	N =	10- 16 y	N =	> 16 y	N =
Lymphocytes (total)	4.1 (1.4-12.1)	10	2.7 (1.4-5.5)	11	2.4 (1.2-4.7)	15	2.4 (1.4-4.2)	15	2.3 (1.2-4.1)	21
T-lymphocytes	2.5 (0.7-8.8)	10	1.9 (0.85-4.3)	11	1.8 (0.77-4.0)	15	1.6 (0.85-3.2)	15	1.5 (0.78-3.0)	21
NK-cells	0.47 (0.055-4.0)	10	0.18 (0.061-0.51)	11	0.20 (0.070-0.59)	15	0.33 (0.092-1.2)	15	0.34 (0.10-1.2)	21
B-lymphocytes	0.76 (0.16-3.7)	10	0.49 (0.18-1.3)	11	0.29 (0.10-0.80)	15	0.30 (0.12-0.74)	15	0.23 (0.064-0.82)	21
Transitional B cells	0.073 (0.0092-0.58)	10	0.054 (0.011-0.27)	10	0.025 (0.0085-0.073)	12	0.016 (0.0029-0.088)	12	0.0077 (0.0006-0.10)	10
Naive B-lymphocytes	0.60 (0.098-3.7)	10	0.34 (0.12-1.0)	11	0.21 (0.070-0.63)	14	0.20 (0.060-0.70)	15	0.12 (0.028-0.55)	16
Natural effector B-lymphocytes	0.041 (0.0046-0.36)	10	0.043 (0.0084-0.22)	11	0.026 (0.0077-0.086)	14	0.022 (0.0082-0.057)	15	0.025 (0.0039-0.17)	16
IgM-only B-lymphocytes	0.0033 (0.0004-0.029)	10	0.0081 (0.0006-0.10)	11	0.0057 (0.0017-0.019)	14	0.0049 (0.0019-0.013)	15	0.0042 (0.0011-0.015)	16
Switched memory B-lymphocytes	0.017 (0.0030-0.098)	10	0.024 (0.0022-0.25)	11	0.019 (0.0070-0.051)	14	0.022 (0.0065-0.073)	15	0.024 (0.0045-0.13)	16
Class switched plasmablasts	0.0049 (0.0008-0.029)	10	0.0032 (0.0003-0.036)	10	0.0020 (0.0007-0.0057)	12	0.0021 (0.0002-0.021)	12	0.0038 (0.0007-0.020)	10
$CD21^{low}/CD38^{low}$ B cells	0.029 (0.0049-0.17)	10	0.026 (0.0069-0.099)	10	0.014 (0.0059-0.036)	12	0.012 (0.0039-0.037)	12	0.0092 (0.0017-0.049)	10
$CD24^{+}/CD38^{+}$ B cells	0.16 (0.020-1.3)	10	0.068 (0.012-0.40)	11	0.031 (0.0067-0.14)	15	0.031 (0.0059-0.17)	15	0.010 (0.0016-0.057)	16
Ig-kappa	0.37 (0.063-2.2)	10	0.26 (0.099-0.68)	11	0.16 (0.066-0.39)	15	0.16 (0.080-0.33)	15	0.12 (0.034-0.39)	21
Ig-lambda	0.29 (0.056-1.5)	10	0.18 (0.069-0.47)	11	0.11 (0.034-0.32)	15	0.11 (0.040-0.31)	15	0.081 (0.024-0.27)	21
Kappa-lambda ratio	1.3 (0.81-2.0)	10	1.4 (0.93-2.2)	11	1.5 (0.99-2.4)	15	1.5 (0.88-2.4)	15	1.4 (1.1-1.8)	21
$CD20^{-}$ B cells	0.0084 (0.0007-0.11)	10	0.0042 (0.0007-0.027)	11	0.0037 (0.0005-0.024)	15	0.0025 (0.0006-0.011)	15	0.0020 (0.0007-0.0062)	16
$CD5^{+}$ B cells	0.34 (0.041-2.7)	10	0.16 (0.023-1.1)	11	0.089 (0.017-0.46)	15	0.095 (0.011-0.85)	15	0.024 (0.0044-0.13)	16
$CD10^{+}$ B cells	0.23 (0.040-1.3)	10	0.11 (0.018-0.63)	11	0.045 (0.010-0.21)	15	0.038 (0.0070-0.20)	15	0.013 (0.0013-0.13)	16

Table 2 Relative numbers of B-lymphocytes in peripheral blood.

Population	N =	Cord blood	N =	1 w – 2 m	N =	2 – 5 m	N =	5 – 9 m	N =	9 – 15 m	N =
T-lymphocytes[a]	136	58 (38-88)	18	70 (55-90)	11	69 (49-97)	12	68 (49-95)	13	70 (56-87)	10
NK-cells[a]	136	22 (8-62)	18	9 (3-23)	11	7 (2-20)	12	9 (2-36)	13	8 (1-64)	10
B-lymphocytes[a]	136	10 (3-30)	18	14 (3-60)	11	17 (8-33)	12	16 (4-54)	13	15 (3-77)	10
Transitional B cells[b]	106	24 (9-66)	11	23 (8-65)	10	14 (4-52)	11	16 (5-50)	10	11 (3-44)	10
Naive B-lymphocytes[b]	127	94 (87-100)	17	95 (90-99)	10	94 (88-100)	11	92 (82-100)	13	92 (88-96)	10
Natural effector B-lymphocytes[b]	127	3 (0.7-10)	17	3 (0.9-10)	10	3 (0.9-11)	11	4 (0.9-16)	13	4 (2-9)	10
IgM-only B-lymphocytes[b]	127	0.1 (0.0-0.6)	17	0.1 (0.0-0.7)	10	0.2 (0.0-2)	11	0.5 (0.1-3)	13	0.4 (0.1-3)	10
Switched memory B-lymphocytes[b]	127	0.7 (0.1-5)	17	0.6 (0.1-5)	10	0.8 (0.1-6)	11	1 (0.3-6)	13	2 (0.7-5)	10
Class switched plasmablasts[b]	106	0.9 (0.1-10)	11	0.7 (0.0-13)	10	0.4 (0.0-6)	11	0.5 (0.0-7)	10	0.3 (0.0-3)	10
$CD21^{low}/CD38^{low}$ B cells[b]	108	3 (0.5-16)	13	2 (0.3-19)	10	3 (0.7-10)	11	4 (0.5-33)	10	3 (0.5-16)	10
$CD24^{+}/CD38^{+}$ B cells[b]	127	40 (12-100)	18	31 (9-100)	10	25 (6-100)	11	23 (7-81)	11	22 (8-61)	10
Ig-kappa[b]	134	51 (43-61)	18	55 (44-67)	10	55 (48-64)	11	54 (45-65)	13	53 (43-66)	10
Ig-lambda[b]	134	45 (37-55)	18	42 (31-58)	10	42 (34-50)	11	43 (35-53)	13	44 (34-57)	10
$CD20^{-}$ B cells[a]	127	0.3 (0.0-7)	18	0.2 (0.0-0.7)	10	0.1 (0.0-0.4)	11	0.2 (0.0-1)	11	0.1 (0.1-0.3)	10
$CD5^{+}$ B cells[b]	127	37 (9-100)	18	50 (14-100)	10	67 (44-100)	11	52 (15-100)	11	52 (18-100)	10
$CD10^{+}$ B cells[b]	127	29 (9-98)	18	41 (24-69)	10	40 (19-85)	11	35 (22-56)	11	29 (14-59)	10

Mean and 90% range. W = week, m = month, y = year. [a] % of total lymphocytes. [b] % of B-lymphocytes.

Population	15 – 24 m	N =	2 -5 y	N =	5 – 10 y	N =	10- 16 y	N =	> 16 y	N =
T-lymphocytes[a]	61 (36-100)	10	69 (52-92)	11	73 (55-97)	15	68 (52-90)	15	67 (50-91)	21
NK-cells[a]	11 (1-96)	10	6 (2-25)	11	8 (2-31)	15	14 (4-51)	15	15 (5-49)	21
B-lymphocytes[a]	19 (8-45)	10	18 (8-39)	11	12 (4-33)	15	13 (7-24)	15	10 (4-28)	21
Transitional B cells[b]	10 (4-28)	10	12 (2-70)	10	8 (2-30)	12	5 (1-25)	12	4 (0.7-24)	10
Naive B-lymphocytes[b]	88 (78-99)	10	73 (39-100)	11	76 (62-94)	14	74 (49-100)	15	63 (33-100)	16
Natural effector B-lymphocytes[b]	6 (2-18)	10	9 (2-43)	11	9 (4-24)	14	8 (2-28)	15	13 (3-61)	16
IgM-only B-lymphocytes[b]	0.5 (0.1-3)	10	2 (0.1-23)	11	2 (0.5-8)	14	2 (0.5-7)	15	2 (0.4-11)	16
Switched memory B-lymphocytes[b]	3 (0.3-20)	10	5 (0.9-29)	11	7 (3-18)	14	8 (1-43)	15	12 (3-46)	16
Class switched plasmablasts[b]	0.7 (0.1-5)	10	0.7 (0.1-4)	10	0.7 (0.1-3)	12	0.7 (0.0-10)	12	2 (0.7-6)	10
$CD21^{low}/CD38^{low}$ B cells[b]	4 (1-17)	10	6 (2-16)	10	5 (1-17)	12	4 (1-11)	12	5 (2-14)	10
$CD24^{+}/CD38^{+}$ B cells[b]	23 (8-69)	10	15 (3-68)	11	11 (4-36)	15	11 (4-33)	15	5 (2-15)	16
Ig-kappa[b]	55 (44-68)	10	57 (47-68)	11	58 (47-73)	15	58 (46-72)	15	58 (52-63)	21
Ig-lambda[b]	43 (34-55)	10	40 (29-54)	11	38 (30-48)	15	40 (30-53)	15	41 (35-47)	21
$CD20^{-}$ B cells[a]	0.2 (0.0-3)	10	0.2 (0.0-0.8)	11	0.2 (0.0-1)	15	0.1 (0.0-0.5)	15	0.1 (0.0-0.2)	16
$CD5^{+}$ B cells[b]	42 (16-100)	10	30 (6-100)	11	30 (13-72)	15	29 (6-100)	15	10 (3-27)	16
$CD10^{+}$ B cells[b]	29 (15-56)	10	20 (5-81)	11	15 (6-37)	15	11 (3-41)	15	5 (1-25)	16

Evaluation of EUROclass classification

The B-lymphocyte subpopulations used in the EUROclass CVID classification clearly show an age-related development during childhood. When our group of healthy children would be assessed according to the EUROclass classification, 40 out of 97 children would be classified in one of the subgroups due to the fact that they show lower relative numbers of switched memory B-lymphocytes (Figure 2). Within this group 27 children showed high relative numbers of transitional B cells. However, 38 of these 40 children were younger than two years of age, when the diagnosis of CVID cannot yet be made.

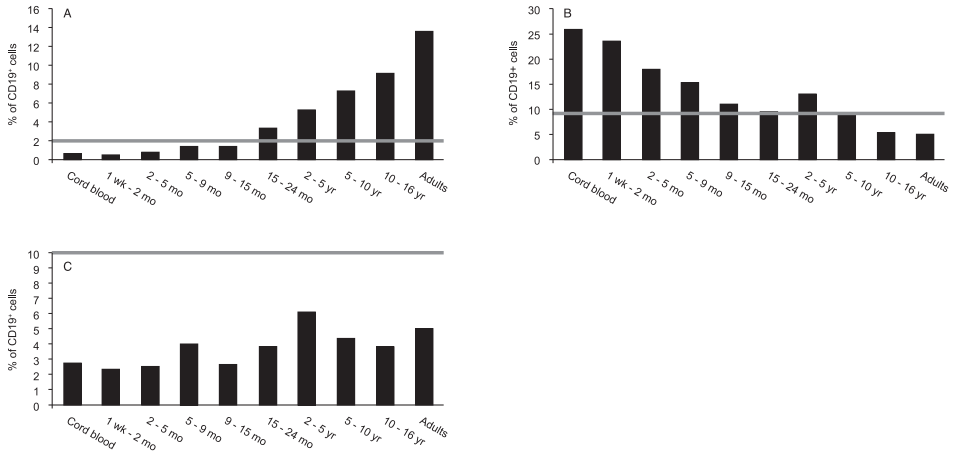

Figure 2

The age-related development of B-lymphocyte subpopulations used in the EUROclass CVID classification (median per age group). (**A**) Switched memory B-lymphocytes ($CD19^+CD27^+IgM^-IgD^-$) with cut-off used in the EUROclass CVID classification (grey line) at 2%. (**B**) Transitional B cells ($CD19^+CD38^{++}IgM^{++}$) with cut-off used in the EUROclass CVID classification (grey line) at 9%. (**C**) $CD21^{low}/CD38^{low}$ B cells ($CD19^+CD38^{low}CD21^{low}$) with cut-off used in the EUROclass CVID classification (grey line) at 10%. All measured as percentage of $CD19^+$ B-lymphocytes in peripheral blood.

Discussion

B cell immunophenotyping characteristics correlate with clinical complications in adults with CVID and are therefore used for classification of patients; currently, the EUROclass classification is most commonly used [17]. We and others [18-26] demonstrate that the B-lymphocyte compartments of normal adults and children of various ages differ considerably, implicating that data obtained in adults should not be extrapolated to children. These differences seem logical when looking at normal B cell development: after initial maturation in the bone marrow, the first cells to emigrate into the peripheral blood are transitional ($CD19^+CD38^{++}IgM^{++}$) B cells and naive B-lymphocytes ($CD19^+CD27^-IgM^+IgD^+$) [29, 30]. The next step leads to short-lived antibody-secreting plasma cells, switched

memory B-lymphocytes, or natural effector cells, depending on the environment during activation, and the presence or absence of help from T-lymphocytes [31–34]. These last steps are antigen dependent; they are also the steps that are disturbed in CVID. CD21^{low} B cells (CD19^{+}CD21^{low}CD38^{low}) are rare in the blood of healthy individuals but can be found in patients with autoimmune disease [35]. Forty out of our 97 normal healthy children could be 'classified' in the CVID-subgroup with the highest risk of complications according to the EUROclass system, but 38 of them were younger than 2 years of age, when the diagnosis of CVID cannot yet be made. A similar pattern is seen in other recently published data of B-lymphocyte subpopulations in healthy children [18]. Two papers have been published examining the EUROclass classification in children with CVID. Van de Ven et al. showed that two of nine children with CVID and heterozygous TACI mutations belonged to the EUROclass high-risk group based on immunophenotyping results (smB-Tr^{high}) [36]. Yong et al. showed the correlation in a small group of children with CVID: children with few or absent switched memory B-lymphocytes (<5/ml; n = 24) exhibited a more severe clinical phenotype and more autoimmune cytopenia (21% vs. 0%) than those with higher numbers of switched memory B-lymphocytes (n = 21) [37]; but this cohort is too small to extrapolate the data to the entire paediatric population. However, the great changes of these populations during development emphasize that a classification developed in adults cannot simply be extrapolated to classify the prognosis of children. A large, multicenter study is needed to evaluate the immunophenotyping characteristics of children with CVID and to correlate these with their clinical phenotype to create a reliable paediatric CVID classification.

Nearly 10% of CVID patients show a disease-modifying mutation in the gene encoding for TACI (TNFRSF13B), a tumour necrosis factor receptor expressed mainly by activated B-lymphocytes (like marginal zone and memory B-lymphocytes), activated T-lymphocytes, monocytes, and dendritic cells. It mediates isotype switching, promotes plasma cell differentiation, and is essential for thymus-independent antibody responses, but also has an inhibitory role in B cell homeostasis [14]. Lack of TACI-expression can be used as a screening method before performing genetic analysis for the gene. There is little information about normal TACI-expression in healthy adults [38], and none in children, however. Plasma levels of BAFF and APRIL (both ligands of TACI) are significantly higher in patients with CVID, and correlate inversely with age in healthy subjects [39], suggesting a positive age effect for TACI. Preterm neonatal naive B-lymphocytes show lower BAFF-R fluorescence intensity compared to adult naive B-lymphocytes, but in the same study no significant difference between TACI-expression on naive B-lymphocytes was found between cord blood and adults [38]. However, a lower gene expression of TACI determined by RT-PCR was seen in preterm cord blood compared to adult blood [38]. We found lower percentages of TACI^{+} B-lymphocytes in younger children compared to older children and adults. We did not find any effect of age on the BAFF-R expression on B-lymphocytes. This means that a low number of TACI-positive B-lymphocytes in young children is not indicative of a potential TACI-mutation.

In conclusion, we established statistically reliable reference ranges of B-lymphocyte subpopulations for children of different ages for all B cell subpopulations relevant in CVID

patients. Although the greatest changes in B-lymphocyte subpopulations occur below the age of 2 years when the diagnosis of CVID cannot yet be made, the development of the peripheral B-lymphocyte population during childhood emphasizes the potential dangers of using a classification developed in adults to classify the prognosis of children and demonstrates the need for a separate paediatric CVID classification.

Acknowledgements
This study was funded by the Peribosch Foundation and the Jeroen Bosch Academie. We would like to thank the laboratory of the Department of Clinical Chemistry and Hematology of the Jeroen Bosch Hospital for their extensive immunophenotyping effort.

References

1. Notarangelo LD, Fischer A, Geha RS, et al. Primary immunodeficiencies: 2009 update. J Allergy Clin Immunol. 2009;124: 1161–78.
2. Geha RS, Notarangelo LD, Casanova JL, et al. Primary immunodeficiency diseases: an update from the International Union of Immunological Societies Primary Immunodeficiency Diseases Classification Committee. J Allergy Clin Immunol. 2007;120:776–94.
3. Ozen A, Baris S, Karakoc-Aydiner E, Ozdemir C, Bahceciler NN, Barlan IB. Outcome of hypogammaglobulinemia in children: immunoglobulin levels as predictors. Clin Immunol 2010;137:374–83.
4. International Union of Immunological Societies. Primary immunodeficiency diseases. Report of an IUIS Scientific Committee. Clin Exp Immunol 1999;118 (Suppl 1):1–28.
5. Hermaszewski RA, Webster AD. Primary hypogammaglobulinaemia: a survey of clinical manifestations and complications. Q J Med 1993;86:31–42.
6. Kutukculer N, Gulez N. The outcome of patients with unclassified hypogammaglobulinemia in early childhood. Pediatr Allergy Immunol 2009;20:693–8.
7. Giovannetti A, Pierdominici M, Mazzetta F, et al. Unravelling the complexity of T cell abnormalities in common variable immunodeficiency. J Immunol 2007;178:3932–43.
8. Cunningham-Rundles C, Bodian C. Common variable immunodeficiency: clinical and immunological features of 248 patients. Clin Immunol. 1999;92:34–48.
9. Touw CM, van de Ven AA, de Jong PA, et al. Detection of pulmonary complications in common variable immunodeficiency. Pediatr Allergy Immunol 2010;21:793–805.
10. Quinti I, Soresina A, Spadaro G, et al. Long-term follow-up and outcome of a large cohort of patients with common variable immunodeficiency. J Clin Immunol 2007;27:308–16.
11. Lucas M, Lee M, Lortan J, Lopez-Granados E, Misbah S, Chapel H. Infection outcomes in patients with common variable immunodeficiency disorders: relationship to immunoglobulin therapy over 22 years. J Allergy Clin Immunol 2010;125:1354–60 e4.
12. van Zelm MC, Reisli I, van der Burg M, et al. An antibody-deficiency syndrome due to mutations in the CD19 gene. N Engl J Med 2006;354:1901–12.
13. Castigli E, Geha RS. Molecular basis of common variable immunodeficiency. J Allergy Clin Immunol 2006;117:740–6; quiz 7.
14. Lee JJ, Ozcan E, Rauter I, Geha RS. Transmembrane activator and calcium-modulator and cyclophilin ligand interactor mutations in common variable immunodeficiency. Curr Opin Allergy Clin Immunol 2008;8:520–6.
15. Piqueras B, Lavenu-Bombled C, Galicier L, et al. Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. J Clin Immunol 2003;23:385–400.
16. Warnatz K, Denz A, Drager R, et al. Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood 2002;99:1544–51.
17. Wehr C, Kivioja T, Schmitt C, et al. The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood 2008;111:77–85.
18. Piatosa B, Wolska-Kusnierz B, Pac M, Siewiera K, Galkowska E, Bernatowska E. B cell subsets in healthy children: reference values for evaluation of B cell maturation process in peripheral blood. Cytometry B Clin Cytom 2010;78:372–81.
19. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, et al. Longitudinal survey of lymphocyte subpopulations in the first year of life. Pediatr Res 2000;47:528–37.
20. Duijts. Infectious Diseases and Immune System in Infants: Risk factors and consequences. The Generation R Study. Thesis 2008. http://www.generationr.nl, accessed August 19, 2011.
21. Erkeller-Yuksel FM, Deneys V, Yuksel B, et al. Age-related changes in human blood lymphocyte subpopulations. J Pediatr 1992;120:216–22.

22. Comans-Bitter WM, de Groot R, van den Beemd R, et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997;130:388–93.
23. Huck K, Feyen O, Ghosh S, Beltz K, Bellert S, Niehues T. Memory B cells in healthy and antibody-deficient children. Clin Immunol 2009;131:50–9.
24. Morbach H, Eichhorn EM, Liese JG, Girschick HJ. Reference values for B cell subpopulations from infancy to adulthood. Clin Exp Immunol 2010;162:271–9.
25. Shearer WT, Rosenblatt HM, Gelman RS, et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol 2003;112:973–80.
26. van Gent R, van Tilburg CM, Nibbelke EE, et al. Refined characterization and reference values of the pediatric T- and B cell compartments. Clin Immunol. 2009;133:95–107.
27. Kraan J, Gratama JW, Keeney M, D'Hautcourt JL. Setting up and calibration of a flow cytometer for multicolor immunophenotyping. J Biol Regul Homeost Agents 2003;17:223–33.
28. Hanh GJ MW. Statistical Intervals: A Guide for Practitioners. Hoboken, NJ: John Wiley and Sons, Inc., 1991.
29. LeBien TW, Tedder TF. B-lymphocytes: how they develop and function. Blood 2008;112:1570–80.
30. Sims GP, Ettinger R, Shirota Y, Yarboro CH, Illei GG, Lipsky PE. Identification and characterization of circulating human transitional B cells. Blood 2005;105:4390–8.
31. Bonilla FA, Oettgen HC. Adaptive immunity. J Allergy Clin Immunol 2010;125:S33–40.
32. Vale AM, Schroeder HW Jr. Clinical consequences of defects in B cell development. J Allergy Clin Immunol 2010;125:778–87.
33 . Bauer K, Zemlin M, Hummel M, et al. Diversification of Ig heavy chain genes in human preterm neonates prematurely exposed to environmental antigens. J Immunol 2002;169:1349–56.
34. Zandvoort A, Timens W. The dual function of the splenic marginal zone: essential for initiation of anti-TI-2 responses but also vital in the general first-line defense against blood-borne antigens. Clin Exp Immunol 2002;130:4–11.
35. Isnardi I, Ng YS, Menard L, et al. Complement receptor 2/CD21human naive B cells contain mostly autoreactive unresponsive clones. Blood 2010;115:5026–36.
36. van de Ven AA, van de Corput L, van Tilburg CM, et al. Lymphocyte characteristics in children with common variable immunodeficiency. Clin Immunol 2009;135:63–71.
37. Yong PL, Orange JS, Sullivan KE. Pediatric common variable immunodeficiency: immunologic and phenotypic associations with switched memory B cells. Pediatr Allergy Immunol 2010;21:852–8.
38. Kaur K, Chowdhury S, Greenspan NS, Schreiber JR. Decreased expression of tumor necrosis factor family receptors involved in humoral immune responses in preterm neonates. Blood 2007;110:2948–54.
39. Jin R, Kaneko H, Suzuki H, et al. Age-related changes in BAFF and APRIL profiles and upregulation of BAFF and APRIL expression in patients with primary antibody deficiency. Int J Mol Med 2008;21:233–8.

CHAPTER 2

Paediatric Reference Values for the Peripheral T Cell Compartment

Scand J Immunol. 2012 Apr;75(4):436-44.

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Abstract

Immunophenotyping of blood lymphocyte subpopulations is an important tool in the diagnosis of immunological and haematological diseases. Paediatric age-matched reference values have been determined for the major lymphocyte populations, but reliable reference values for the more recently described T-lymphocyte subpopulations, like different types of memory T-lymphocytes, recent thymic emigrants, regulatory T cells and $CXCR5^+$ helper T-lymphocytes, are not sufficiently available yet. We determined reference values for the absolute and relative sizes of T-lymphocyte subpopulations in healthy children using the lysed whole blood method, which is most often used in diagnostic procedures. When the absolute numbers of some or all T-lymphocyte subpopulations fall outside these reference ranges, this may indicate disease. The reference values show the course of T-lymphocyte development in healthy children. Absolute T-lymphocyte numbers increase 1.4-fold during the first months of life, and after 9–15 months, they decrease threefold to adult values; this is mainly caused by the expansion of recent thymic emigrants and naive cells. Helper and cytotoxic T-lymphocytes show the same pattern. Regulatory T cells increase in the first 5 months of life and then gradually decrease to adult values, although the absolute numbers remain small. The relative number of $CXCR5^+$ cells within the $CD4^+CD45RO^+$ T-lymphocytes increases during the first 6 months of life and then remains more or less stable around 20%.

Introduction

Immunophenotyping of blood lymphocyte subpopulations is an important tool in the diagnosis of immunological and haematological diseases, also in children. Paediatric age-matched reference values have been determined for the major lymphocyte populations (B-, T-, helper T- and cytotoxic T-lymphocytes, and NK cells) [1–4]. These studies reported an increase in the absolute number of $CD3^+$ T-lymphocytes immediately after birth and a gradual decrease from 2 years of age to adulthood. $CD3^+CD4^+$ helper T-lymphocytes showed the same pattern, but $CD3^+CD8^+$ cytotoxic T-lymphocytes were reported to remain stable up to 2 years of age and then to show a gradual decrease towards adult levels. Absolute numbers of NK cells decrease rapidly during the first 2 months of life and continue to gradually decrease thereafter.

In the past decade, new insights in the composition of the peripheral T cell compartment have revealed several 'new' subpopulations, like different types of memory T-lymphocytes [5–7], recent thymic emigrants [8, 9], regulatory T cells [10] and $CXCR5^+$ helper T-lymphocytes [11]. In the maturing immune system of children, these subsets are of special interest; they support the diagnostic process and in some cases, the evaluation of treatment strategies of immunological diseases in children [12]. Van Gent et al. [13] recently used thawed, cryopreserved, isolated peripheral blood mononuclear cells to determine different T-lymphocyte subpopulations in children, but this technique can affect the composition and phenotype of (subpopulations of) the peripheral lymphocyte compartment [14, 15]. Hence, these data are not suitable as reference values for lysed whole blood testing results, the technique most commonly used in routine diagnosis.

Therefore, we determined reference values for T-lymphocyte subpopulations in healthy children using the lysed whole blood method.

Methods

Subjects and samples

Leftover ethylenediaminetetraaceticacid (EDTA) blood from healthy children, who underwent venipuncture or blood sampling by heel prick or finger prick for other reasons, was used for the study. We also asked parents of otherwise healthy infants visiting the paediatric outpatient clinic permission to perform a venipuncture, heel prick or finger prick for study purposes only; after informed consent, 1–2 ml of EDTA blood was taken. Neonatal cord blood was obtained by venipuncture immediately after clamping of the cord. Patients with an active infection, diseases of the immune system or on immunosuppressive therapy were excluded. Below 2 years of age, patients with perinatal problems such as prematurity (gestational age <35 weeks), birthweight <p10 or >p90, congenital or perinatal infection, artificial delivery, congenital deformities and suspected metabolic or neurological disease were also excluded. The study population was divided into ten age groups according to Comans-Bitter et al.[2]: neonatal cord blood (group 1),

1 week–2 months (group 2), 2–5 months (group 3), 5–9 months (group 4), 9–15 months (group 5), 15–24 months (group 6), 2–5 years (group 7), 5–10 years (group 8), 10–16 years (group 9) and 16 years and older (group 10). Blood samples were obtained between April 2008 and January 2011. This study was approved by the local Medical Ethics Committee.

Flow cytometric analysis

Four-colour flow cytometric immunophenotyping with directly labelled monoclonal antibodies (MAb) was used to determine the following lymphocyte subpopulations: T-lymphocytes ($CD3^+$), B-lymphocytes ($CD19^+$), natural killer (NK) cells ($CD3^-CD16^+$ and/or $CD56^+$), helper T-lymphocytes ($CD3^+CD4^+$), cytotoxic T-lymphocytes ($CD3^+CD8^+$), NK-T cells ($CD3^+CD16^+$and/or$CD56^+$), naive helper T-lymphocytes ($CD3^+CD4^+CD45RA^+CD27^+$), terminally differentiated helper T-lymphocytes ($CD3^+CD4^+CD45\ RA^+CD27^-$), central memory helper T-lymphocytes ($CD3^+CD4^+CD45RA^-CD27^+$), effector memory helper T-lymphocytes ($CD3^+CD4^+CD45RA^-CD27^-$), naive cytotoxic T-lymphocytes ($CD8^+CD45RA^+CD197^+CD27^+$), terminally differentiated cytotoxic T-lymphocytes ($CD8^+CD45RA^+CD197^-CD27^-$), central memory cytotoxic T-lymphocytes ($CD8^+CD45RA^-CD197^+CD27^+$), effector memory cytotoxic T-lymphocytes ($CD8^+CD45RA^-CD197^-CD27^-$) [16], recent thymic emigrants ($CD3^+CD4^+CD45RA^+CD31^+$) and regulatory T cells ($CD3^+CD4^+CD25^{++}CD127^-$). We also analysed the expression of T cell receptor (TCR)-αβ and TCR-γδ on the $CD4^+$ and on the $CD8^+$ T-lymphocytes, as well as the expression of CD185 (=CXCR5) on $CD3^+CD4^+CD45RO^+$ T-lymphocytes.

Aliquots were incubated for 15 min in the dark at room temperature with a mixture of optimally titrated MAbs within 24 h after sampling. The antibodies used were CD3 fluoresceïne-isothiocyanate (FITC), CD45RA FITC, TCR- αβ FITC, CD4 phycoerythrin (PE), CD16 PE, CD31 PE, CD56 PE, CD127 PE, CD197 PE (=CCR7), TCR- γδ PE, CD3 peridinin chlorophyll protein-cyanin (PerCP-Cy-5.5), CD4 PerCP-Cy-5.5, CD8 PerCP-Cy-5.5, CD19 PerCP-Cy-5.5, CD45 PerCP-Cy-5.5, CD4 allophycocyanin (APC), CD14 APC, CD25 APC, CD27 APC, CD45RO APC, CD185 Alexa Fluor®488 (all Becton Dickinson, San Jose, CA, USA), CD235a FITC and CD71 PE (Sanquin, Amsterdam, the Netherlands).

Before surface staining, erythrocytes were lysed with ammonium chloride (NH_4Cl). Remaining cells were washed twice with phosphate-buffered saline/bovine serum albumin 0.5% and analysed with a FACSCalibur flow cytometer (Becton Dickinson) using CELLQUESTPRO software (Becton Dickinson). Calibration of the flow cytometer took place with CaliBRITE beads according to the manufacturer's instructions (Becton Dickinson) and daily quality control with Cyto-Cal (microgenics Duke Scientific, Fremont CA, USA) following the guidelines of Kraan et al. [17].

The lymphogate was checked with a CD3/CD14 labelling and considered correct if <1% monocyte contamination was present. B-lymphocytes and NK cells were used to check the 'lymphosum' (B + T + NK = 100 $\pm$ 5%). Leucocyte count and differential were determined with a routine hematology analyzer (XE 2100; Sysmex, Kobe, Japan). In neonatal cord blood, the lymphogate was corrected % of lymphocyte subpopulation

= % of lymphocyte subpopulation within the lymphogate x 100/(100 - [%$CD71^+$ normoblasts + %$CD235^+CD71^-$ unlysed erythrocytes within the lymphogate]). The absolute size of each lymphocyte subpopulation was calculated by multiplying the relative size of the lymphocyte subpopulation and the absolute lymphocyte count.

Statistics

The number of subjects in the different age groups varied between 8 and 21 per tested subpopulation, numbers that are too low to determine robust percentile points at 5% and 95%. Confidence intervals may seem to offer an alternative, but deal with estimating the range of the population mean, and do not cover the distribution of the population values. The proper statistical procedure is to calculate the tolerance interval, which enclosures a specific proportion of the population, estimated on the basis of the values sampled. The tolerance interval takes into account the sample size, the noise in the estimates of the mean and standard deviation, and the confidence about the tolerance interval [18]. We set the proportion to be included at 0.90 (two-sided, comparable to the percentile points p5 and p95), with a confidence level of 0.95.

Tolerance intervals assume normally distributed populations. Most of our data were positively skewed. In 75% of the cases, the means were larger than the median (426 of 570 subsets of measurements = 57 sets of measurements in 10 age groups). Logarithmic transformation reduced the number of cases to 43% (244), which is much closer to the expected percentage (50%). Two tests of normality were applied to each subset of the 57 measurements: the Kolmogorov–Smirnov test and the Shapiro– Wilks test. The original values returned 135 (24%) and 180 (32%) significant violations ($\alpha = 0.05$) of the normality assumption. The log-transformed values returned 64 (11%) and 73 (13%) violations, which is far more close to the expected percentage (5%). The logarithmic transformation has the additional advantage that the estimated tolerance intervals do not include non-existing negative values. However, after logarithmic transformation, the tolerance intervals sometimes seemed to be rather broad, in particular in the case of the relative numbers. This is explained by the large standard deviations in these groups with very low concentration levels, which easily lead to measurement errors and extreme differences between the low and high values in the measurements. In 30 cases, the ratio of the highest and lowest values of the logarithmic standard deviations was >1.100. In these cases, the right limit of the tolerance interval was based on the original values, as indicated by an asterisk in the tables. All other values given in the tables are the retransformed logarithmic values. To evaluate the age effect in the 57 sets of measurements, a one-way ANOVA test ($\alpha = 0.05$) was applied. All calculations and tests were performed with SPSS 16.0 for Windows (IBM, Armonk, NY, USA).

Results

The absolute and relative sizes of the measured T-lymphocyte subpopulations are shown in Tables 1 and 2. All sets of measurements showed a statistically significant age effect ($\alpha = 0.05$), except for absolute and relative values of terminally differentiated helper T-lymphocytes, possibly due to the small numbers of this subpopulation, and for the relative values of regulatory T cells.

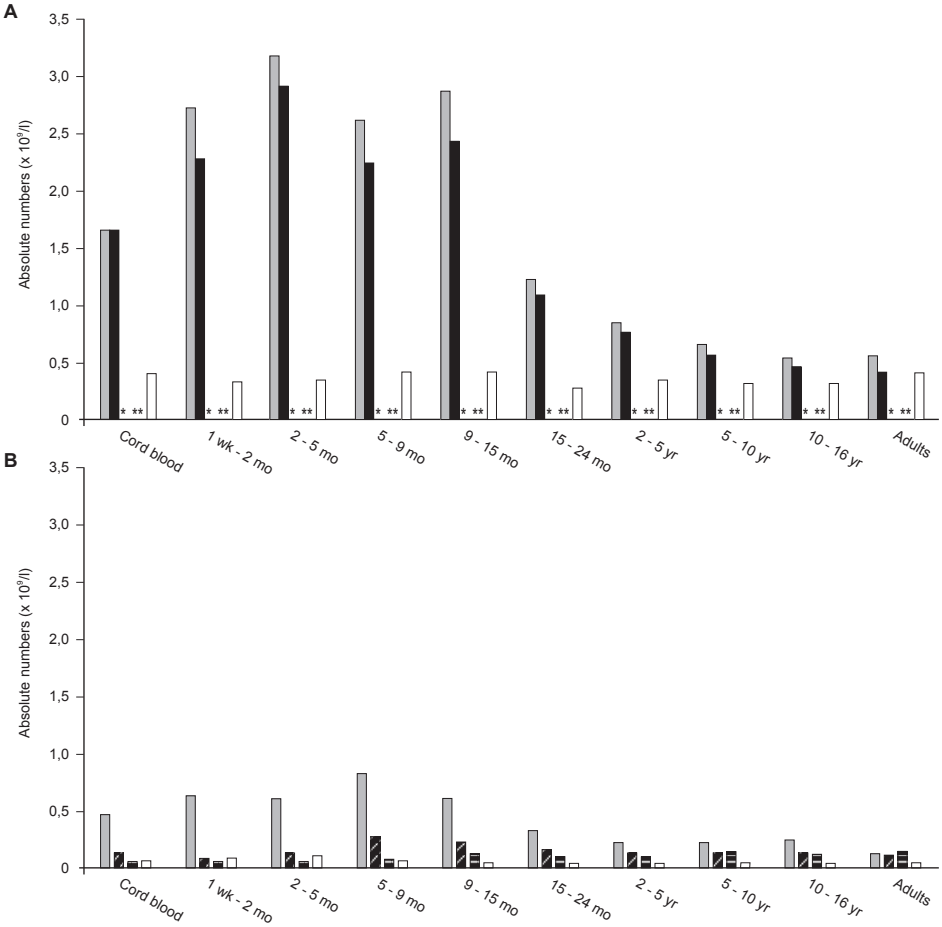

Figure 1
Composition of the T-lymphocyte compartment during childhood (median per age group). (**A**) naive helper T cells ($CD3^+CD4^+CD27^+CD45RA^+$) (grey bars), recent thymic emigrants ($CD3^+CD4^+CD45RA^+CD31^+$) (black bars), terminally differentiated helper T cells ($CD3^+CD4^+CD27^-CD45RA^+$) (bars with *), effector memory helper T cells ($CD3^+CD4^+CD27^-CD45RA^-$) (bars with **) and central memory helper T cells ($CD3^+CD4^+CD27^+CD45RA^-$) (white bars). (**B**) naive cytotoxic T cells ($CD3^+CD8^+CD45RA^+CD197^+$) (grey bars), terminally differentiated cytotoxic T cells ($CD3^+CD8^+CD45RA^+CD197^-$) (diagonal striped bars), effector memory cytotoxic T cells ($CD3^+CD8^+CD45RA^-CD197^-$) (horizontal striped bars) and central memory cytotoxic T cells ($CD3^+CD8^+CD45RA^-CD197^+$) (white bars).

Absolute T-lymphocyte numbers increase 1.4-fold during the first months of life, and after 9–15 months, they decrease threefold to adult values. NK cells show more than 50% decrease of absolute numbers immediately after birth, with a more gradual decrease until 2– 5 years of age and a small increase in later childhood. Helper T-lymphocytes as well as cytotoxic T-lymphocytes show the same pattern as total T-lymphocytes with an increase in the first months of life and a decline after 9– 15 months of age. The expansion of total T-lymphocytes is caused mainly by naive helper T-lymphocytes, although naive cytotoxic T-lymphocytes also almost double during the first 15 months of life, but their numbers are smaller (Figure 1). Central memory helper T-lymphocytes are already present at birth and remain relatively stable, whereas effector memory helper T-lymphocytes are almost absent at birth and gradually increase in later years. Absolute counts of terminally differentiated helper T-lymphocytes remain extremely low. Memory cytotoxic T-lymphocytes are already present at birth; central memory cytotoxic T-lymphocytes follow the pattern of expansion and then gradual decrease, whereas effector memory T-lymphocytes show expansion until adulthood. Terminally differentiated cytotoxic T-lymphocytes form a substantial part of the total peripheral cytotoxic T-lymphocyte population and follow the pattern of expansion and then gradual decrease. Recent thymic emigrants increase 1.6-fold in the first 5 months of life and then gradually decrease almost 8-fold to adult values, not unexpectedly following the pattern of naive helper T-lymphocytes (Figure 1).

Absolute numbers of regulatory T cells show a comparable pattern to naive helper T-lymphocytes, but their absolute numbers remain small. Their relative number within the total peripheral $CD4^+$ T-lymphocyte population remains stable during life (Figure 2). The relative number of $CXCR5^+$ cells within the $CD4^+CD45RO^+$ T-lymphocyte subset increases during the first 6 months of life and then remains more or less stable around 20%. The absolute numbers of $CD3^+CD4^+CD45RO^+CXCR5^+$ cells increase during childhood until adult values are reached (Figure 3).

Discussion

In this study, we confirm – in accordance with previous publications [1–4, 13] – that the T-lymphocyte compartments of normal adults and children of various ages differ considerably. When studying the composition of the T-lymphocyte compartment in children for research purposes as well as in a diagnostic setting, it is important to realize this.

Our results concerning the age distribution of absolute numbers of cytotoxic T-lymphocytes differ from previous studies, which report stable numbers during the first months of life, however; we found a clear increase of cytotoxic T-lymphocyte numbers until 9–15 months of age and a decrease thereafter, similar to the pattern found for helper T-lymphocytes. Helper T-lymphocyte and cytotoxic T-lymphocyte both show a massive increase of the naive population in early years, but there is a clear difference between the subset differentiation of helper and cytotoxic T-lymphocytes with increasing age.

The central memory pool of helper T-lymphocytes remains relatively stable over the years, and the effector memory and terminally differentiated subsets remain very small in number, but central memory and terminally differentiated cytotoxic T-lymphocyte double during the first months of life and then gradually decrease, whereas the effector memory subset shows increasing numbers with age. Our age-related reference values for these subpopulations can be used to help discern normal from abnormal T-lymphocyte development in children.

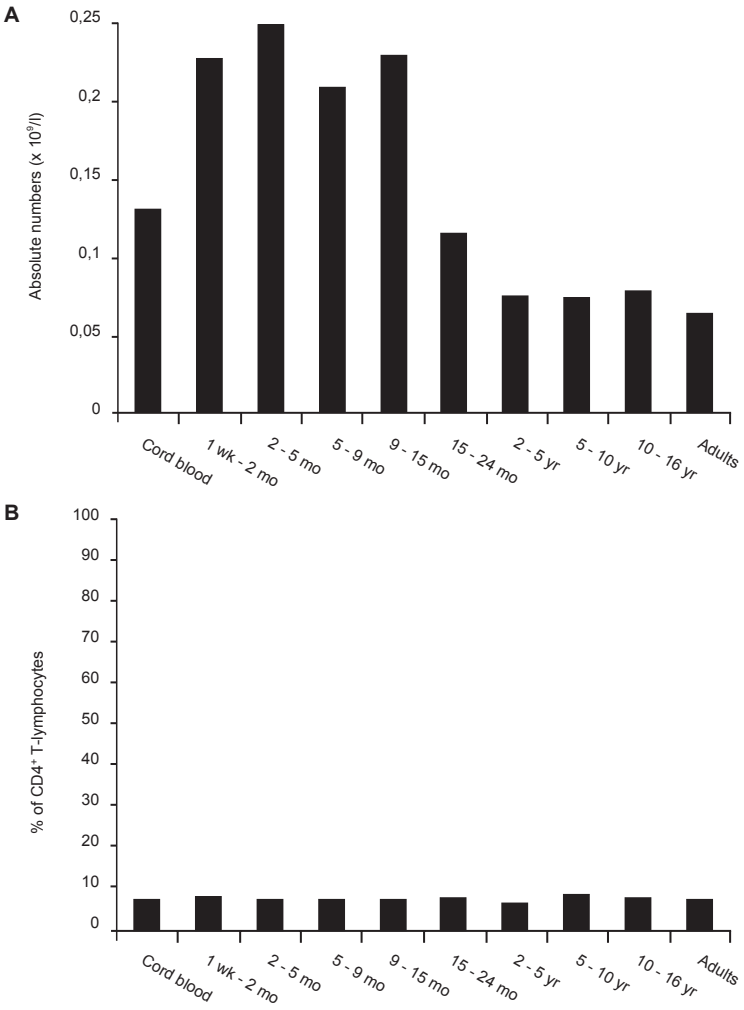

Figure 2
Regulatory T cells during childhood (median per age group). (**A**) absolute numbers of regulatory T cells ($CD3^+CD4^+CD25^{++}CD127^-$). (**B**) relative numbers of regulatory T cells ($CD3^+CD4^+CD25^{++}CD127^-$) within $CD4^+$ T-lymphocytes.

The neonatal CD4+ compartment contains more recent thymic emigrants than the adult CD4+ compartment [8, 9]. Not unexpectedly, our data show that recent thymic emigrants follow the pattern of naive helper T-lymphocytes after the neonatal period (Figure 1). Recent thymic emigrants are not only interesting when exploring the maturing immune system, abnormal numbers are also associated with several diseases: decreased numbers of recent thymic emigrants are seen in children with rheumatoid factor–negative polyarticular juvenile idiopathic arthritis and in adults with SLE and psoriasis [19–21],

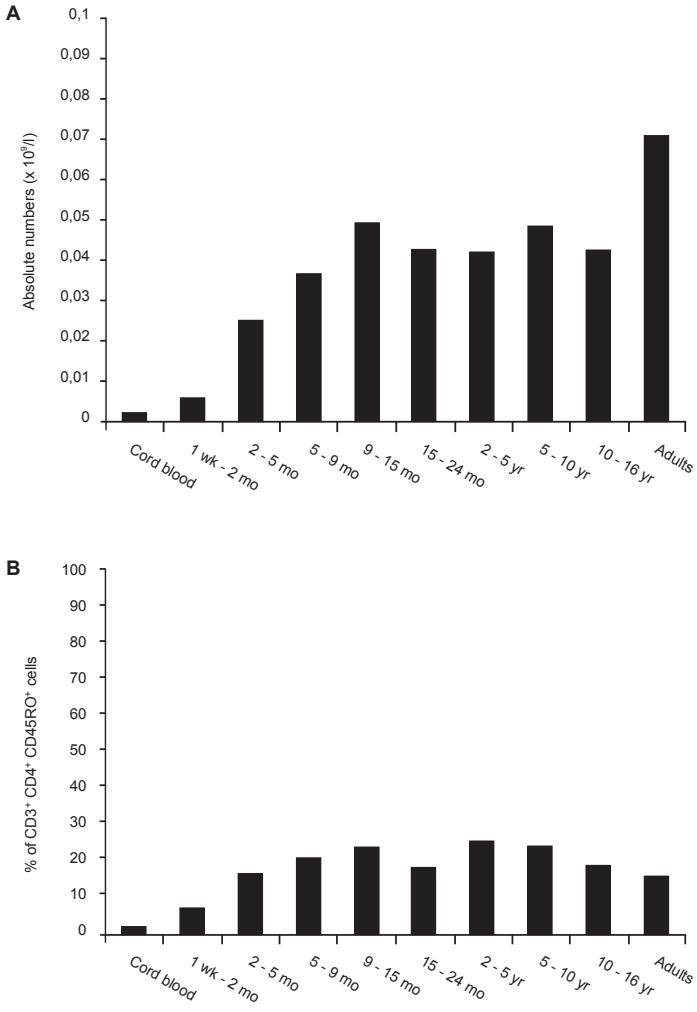

Figure 3
CXCR5+ memory helper T-lymphocytes during childhood (median per age group). (**A**) absolute numbers of CXCR5+ memory helper T-lymphocytes (CD3+CD4+CD45RO+CXCR5+). **(B**) relative numbers of CXCR5+ cells within CD4+CD45RO+ memory T-lymphocytes.

whereas type I diabetes is associated with increased recent thymic emigrants in children and adolescents [22].

Dysfunction of regulatory T cells is associated with autoimmune disease, immunopathology and allergy [23]. Several primary immunodeficiency diseases are associated with abnormalities of regulatory T cells; one example is 'immune dysregulation, polyendocrinopathy, enteropathy, X-linked' [24]. Also, subgroups of common variable immunodeficiency (CVID) patients show decreased numbers of regulatory T cells, associated with specific clinical phenotypes [25]. Especially when exploring the latter in children, it is important to evaluate the results using age-related reference values (Figure 2).

The CXC chemokine receptor 5 (CXCR5) is widely expressed on B-lymphocytes and is required for the development of follicles in secondary lymphoid tissue [26]. CXCR5 is also expressed on a subset of circulating memory helper T-lymphocytes in the germinal centres of secondary lymphoid organs where they efficiently support the production of immunoglobulins IgA and IgG [11, 27, 28]. Interestingly, $CXCR5^+$ memory T-lymphocytes are virtually absent at birth and increase in numbers during the first year of life, paralleling the start of infantile IgA and IgG production (Figure 3).

In conclusion, we determined age-related reference values for the peripheral T-lymphocyte compartment, including several more recently described subpopulations, using the lysed whole blood method and the statistical method of tolerance intervals to obtain reliable data that efficiently use all the information available. When absolute numbers of some or all T-lymphocyte subpopulations fall outside these reference ranges, this may indicate disease. Our data enable evaluation of these T-lymphocyte subpopulations in primary immunodeficiencies and auto-immune diseases in childhood.

Acknowledgment

This study was funded by the Peribosch Foundation and the Jeroen Bosch Academy. We would like to thank Dr Mirjam van der Burg from the Department of Immunology of the Erasmus Medical Centre in Rotterdam, the Netherlands, for her advice, and the laboratory of the Department of Clinical Chemistry and Hematology of the Jeroen Bosch Hospital for their extensive immunophenotyping effort.

Table 1 Absolute numbers of T-lymphocytes.

Population	N =	Cord blood	N =	1 w – 2 m	N =	2 – 5 m	N =	5 – 9 m	N =	9 – 15 m	N =
Lymphocytes (total)	136	5.4 (3.1-9.4)	18	5.7 (2.9-11.4)	11	6.5 (3.4-12.2)	12	5.8 (1.8-18.7)	13	6.3 (3.2-12.3)	10
B-lymphocytes	136	0.54 (0.14-2.0)	18	0.81 (0.18-3.5)	11	1.1 (0.52-2.3)	12	0.90 (0.13-6.3)	13	0.94 (0.11-7.7)	10
NK-cells	136	1.2 (0.5-3.1)	18	0.51 (0.14-1.9)	11	0.44 (0.097-1.99)	12	0.52 (0.068-3.9)	13	0.50 (0.071-3.5)	10
T-lymphocytes	136	3.1 (1.4-6.8)	18	4.0 (1.9-8.4)	11	4.5 (2.2-9.2)	12	4.0 (1.4-11.5)	13	4.4 (2.4-8.3)	10
Helper T-lymphocytes	136	2.2 (1.0-4.8)	18	3.0 (1.5-6.0)	11	3.3 (1.6-6.5)	12	2.7 (1.0-7.2)	13	3.0 (1.3-7.1)	10
Cytotoxic T-lymphocytes	136	0.8 (0.2-2.7)	18	0.9 (0.3-2.7)	11	1.0 (0.3-3.4)	12	1.1 (0.2-5.4)	13	1.2 (0.4-4.1)	10
Naïve helper- cells	125	1.8 (0.9-3.9)	18	2.7 (1.3-5.7)	10	3.1 (1.6-6.0)	11	2.5 (0.8-7.6)	11	2.7 (1.1-6.4)	10
Terminally differentiated helper T cells	125	0.00079 (0.00021-0.003)	18	0.0005 (0.00017-0.0015)	10	0.0011 (0.00024-0.0053)	11	0.0027 (0.0000047-0.4)*	11	0.0021 (0.0000081-0.077)*	10
Effector memory helper T cells	125	0.00067 (0.00017-0.0026)	18	0.0017 (0.00011-0.026)	10	0.0064 (0.002-0.021)	11	0.0093 (0.0012-0.072)	11	0.013 (0.00043-0.12)*	10
Central memory helper T cells	125	0.39 (0.075-2.0)	18	0.39 (0.09-1.7)	10	0.35 (0.053-2.2)	11	0.32 (0.083-1.3)	11	0.39 (0.19-0.8)	10
Naïve cytotoxic T cells	125	0.36 (0.023-1.3)*	18	0.55 (0.14-2.2)	10	0.69 (0.29-1.65)	11	0.7 (0.15-3.2)	11	0.58 (0.14-2.46)	10
Terminally differentiated cytotoxic T- cells	125	0.095 (0.012-0.75)	18	0.086 (0.015-0.48)	10	0.1 (0.013-0.82)	11	0.22 (0.017-2.8)	11	0.22 (0.029-1.7)	10
Effector memory cytotoxic T cells	125	0.044 (0.0074-0.26)	18	0.042 (0.0094-0.19)	10	0.056 (0.0024-0.4)*	11	0.1 (0.0076-1.4)	11	0.13 (0.0076-2.1)	10
Central memory cytotoxic T cells	125	0.04 (0.0011-0.16)*	18	0.071 (0.013-0.38)	10	0.059 (0.0006-0.19)*	11	0.037 (0.0018-0.15)*	11	0.034 (0.012-0.092)	10
CD197 +/- cytotoxic T cells	125	0.56 (0.13-2.4)	18	0.65 (0.18-2.3)	10	0.81 (0.31-2.1)	11	0.96 (0.18-5.0)	11	0.86 (0.25-3.0)	10
Recent thymic emigrants	126	1.7 (0.71-4.2)	18	2.2 (1.0-4.9)	10	2.7 (1.4-5.2)	11	2.2 (0.8-6.2)	11	2.3 (0.9-5.8)	10
NK-T cells	136	0.034 (0.0056-0.21)	18	0.025 (0.0069-0.091)	11	0.035 (0.013-0.09)	12	0.047 (0.0044-0.51)	13	0.049 (0.0075-0.33)	10
$CXCR5^+$ memory helper T cells	102	0.0019 (0.00052-0.0066)	14	0.0063 (0.00067-0.06)	10	0.024 (0.0062-0.089)	11	0.041 (0.015-0.11)	10	0.048 (0.011-0.2)	10
Regulatory T cells	124	0.144 (0.065-0.319)	18	0.23 (0.083-0.64)	10	0.25 (0.12-0.53)	11	0.21 (0.06-0.74)	11	0.21 (0.063-0.69)	10
TCR- alpha/beta	127	2.8 (1.1-6.9)	18	3.7 (1.6-8.6)	10	4.3 (2.2-8.3)	11	3.8 (1.2-12.0)	11	4.0 (2.0-8.0)	10
Double negative T cells	123	0.028 (0.01-0.079)	18	0.022 (0.0055-0.086)	10	0.034 (0.0082-0.14)	11	0.046 (0.016-0.14)	11	0.04 (0.012-0.14)	10
Helper T cells	123	1.9 (0.8-4.7)	18	2.8 (1.2-6.2)	10	3.1 (1.6-6.0)	11	2.5 (0.83-7.8)	11	2.7 (1.0-7.0)	10
Cytotoxic T cells	123	0.73 (0.23-2.4)	18	0.85 (0.23-3.1)	10	1.1 (0.4-2.8)	11	1.2 (0.27-5.2)	11	1.2 (0.36-3.8)	10
Double positive T cells	123	0.039 (0.0073-0.21)	18	0.049 (0.015-0.17)	10	0.059 (0.014-0.24)	11	0.035 (0.0063-0.19)	11	0.027 (0.0066-0.11)	10
TCR- gamma/delta	127	0.087 (0.03-0.25)	18	0.12 (0.013-1.0)	10	0.17 (0.056-0.51)	11	0.18 (0.038-0.89)	11	0.21 (0.07-0.63)	10
Double negative T cells	123	0.059 (0.02-0.17)	18	0.075 (0.0077-0.73)	10	0.094 (0.026-0.34)	11	0.12 (0.032-0.43)	11	0.14 (0.047-0.43)	10
Helper T cells	123	0.014 (0.0034-0.061)	18	0.02 (0.0039-0.1)	10	0.03 (0.01-0.086)	11	0.017 (0.0056-0.051)	11	0.013 (0.00092-0.17)	10
Cytotoxic T cells	123	0.011 (0.0027-0.046)	18	0.012 (0.00025-0.14)*	10	0.04 (0.012-0.13)	11	0.043 (0.004-0.45)	11	0.046 (0.008-0.27)	10
Double positive T cells	123	0.0005 (0.000098-0.0026)	18	0.00082 (0.00012-0.0057)	10	0.0021 (0.00029-0.015)	11	0.0011 (0.000097-0.012)	11	0.0058 (0.000067-0.005)	10

Mean and 90% range x 10^9/l. W = week, m = month, y = year. * The right limit of this tolerance interval was based on the original values (see Method). ** Age range: 16 – 76 years. Median age: 33 years.

Population	15 – 24 m	N =	2 -5 y	N =	5 – 10 y	N =	10- 16 y	N =	> 16 y**	N =
Lymphocytes (total)	4.1 (1.4-12.1)	10	2.7 (1.4-5.5)	11	2.4 (1.2-4.7)	15	2.4 (1.4-4.2)	15	2.3 (1.2-4.1)	21
B-lymphocytes	0.76 (0.16-3.7)	10	0.49 (0.18-1.3)	11	0.29 (0.10-0.80)	15	0.30 (0.12-0.74)	15	0.23 (0.064-0.82)	21
NK-cells	0.47 (0.055-4.0)	10	0.18 (0.061-0.51)	11	0.20 (0.070-0.59)	15	0.33 (0.092-1.2)	15	0.34 (0.10-1.2)	21
T-lymphocytes	2.5 (0.7-8.8)	10	1.9 (0.85-4.3)	11	1.8 (0.77-4.0)	15	1.6 (0.85-3.2)	15	1.5 (0.78-3.0)	21
Helper T-lymphocytes	1.6 (0.4-7.2)	10	1.1 (0.5-2.7)	11	1.0 (0.4-2.5)	15	0.9 (0.4-2.1)	15	1.0 (0.5-2.0)	21
Cytotoxic T-lymphocytes	0.7 (0.2-2.8)	10	0.6 (0.2-1.8)	11	0.6 (0.2-1.7)	15	0.6 (0.3-1.3)	15	0.5 (0.2-1.2)	21
Naïve helper- cells	1.3 (0.2-7.5)	10	0.8 (0.3-2.3)	11	0.7 (0.2-2.5)	13	0.6 (0.2-1.7)	15	0.5 (0.1-2.3)	16
Terminally differentiated helper T cells	0.0015 (0.0001-0.033)*	10	0.0017 (0.000061-0.016)*	11	0.0011 (0.000025-0.025)*	13	0.0016 (0.00004-0.051)*	15	0.0037 (0.000098-0.068)*	16
Effector memory helper T cells	0.011 (0.00085-0.15)	10	0.018 (0.0035-0.089)	11	0.023 (0.003-0.17)	13	0.033 (0.0051-0.21)	15	0.053 (0.013-0.22)	16
Central memory helper T cells	0.17 (0.001-0.65)*	10	0.33 (0.16-0.66)	11	0.18 (0.0037-0.51)*	13	0.3 (0.12-0.74)	15	0.43 (0.18-1.1)	16
Naïve cytotoxic T cells	0.31 (0.03-3.1)	10	0.24 (0.053-1.1)	11	0.24 (0.042-1.3)	13	0.22 (0.078-0.64)	15	0.13 (0.016-1.0)	16
Terminally differentiated cytotoxic T- cells	0.16 (0.055-0.46)	10	0.12 (0.025-0.53)	11	0.14 (0.057-0.34)	13	0.12 (0.035-0.42)	15	0.084 (0.025-0.28)	16
Effector memory cytotoxic T cells	0.1 (0.016-0.63)	10	0.12 (0.024-0.59)	11	0.14 (0.045-0.41)	13	0.11 (0.016-0.81)	15	0.16 (0.04-0.64)	16
Central memory cytotoxic T cells	0.022 (0.0048-0.11)	10	0.017 (0.0043-0.064)	11	0.016 (0.0061-0.043)	13	0.014 (0.0023-0.086)	15	0.024 (0.0047-0.12)	16
CD197 +/- cytotoxic T cells	0.5 (0.1-2.4)	10	0.37 (0.12-1.2)	11	0.38 (0.1-1.5)	13	0.36 (0.16-0.8)	15	0.23 (0.06-0.83)	16
Recent thymic emigrants	1.1 (0.17-7.4)	10	0.71 (0.19-2.6)	11	0.59 (0.2-1.7)	14	0.48 (0.15-1.5)	15	0.34 (0.05-2.4)	16
NK-T cells	0.038 (0.0065-0.23)	10	0.06 (0.015-0.25)	11	0.064 (0.012-0.34)	15	0.075 (0.016-0.35)	15	0.096 (0.023-0.41)	21
$CXCR5^+$ memory helper T cells	0.045 (0.015-0.14)	10	0.048 (0.013-0.17)	9	0.047 (0.013-0.16)	8	0.047 (0.014-0.16)	10	0.067 (0.024-0.19)	10
Regulatory T cells	0.12 (0.02-0.77)	10	0.076 (0.039-0.15)	11	0.073 (0.02-0.27)	15	0.078 (0.033-0.19)	12	0.067 (0.025-0.18)	16
TCR- alpha/beta	2.2 (0.6-8.5)	10	1.6 (0.6-4.3)	11	1.5 (0.6-3.7)	15	1.4 (0.7-2.8)	15	1.4 (0.6-3.3)	16
Double negative T cells	0.033 (0.011-0.1)	10	0.047 (0.016-0.14)	11	0.032 (0.01-0.1)	15	0.027 (0.009-0.078)	14	0.023 (0.0069-0.074)	13
Helper T cells	1.4 (0.3-7.0)	10	1.0 (0.4-2.8)	11	0.91 (0.36-2.8)	15	0.85 (0.34-2.1)	14	0.85 (0.3-2.4)	13
Cytotoxic T cells	0.65 (0.17-2.5)	10	0.54 (0.17-1.7)	11	0.57 (0.21-1.5)	15	0.54 (0.21-1.5)	14	0.45 (0.12-1.7)	13
Double positive T cells	0.014 (0.0023-0.088)	10	0.012 (0.0033-0.043)	11	0.0081 (0.0036-0.018)	15	0.0091 (0.0032-0.026)	14	0.012 (0.0023-0.06)	13
TCR- gamma/delta	0.16 (0.041-0.64)	10	0.16 (0.027-0.96)	11	0.16 (0.027-0.96)	15	0.15 (0.039-0.54)	15	0.071 (0.025-0.2)	16
Double negative T cells	0.12 (0.027-0.5)	10	0.13 (0.021-0.85)	11	0.13 (0.033-0.54)	15	0.13 (0.039-0.44)	14	0.056 (0.019-0.17)	13
Helper T cells	0.008 (0.00087-0.073)	10	0.0047 (0.001-0.021)	11	0.0028 (0.00051-0.015)	15	0.0022 (0.00041-0.012)	14	0.001 (0.00012-0.0076)	13
Cytotoxic T cells	0.03 (0.005-0.19)	10	0.019 (0.0019-0.2)	11	0.019 (0.0051-0.069)	15	0.02 (0.0021-0.19)	14	0.013 (0.0018-0.094)	13
Double positive T cells	0.00041 (0.000038-0.0045)	10	0.0003 (0.000038-0.0045)	11	0.0002 (0.000034-0.0011)	15	0.0002 (0.00002-0.0021)	14	0.0002 (0.000035-0.0011)	13

Table 2 Relative numbers of T-lymphocytes.

Population	N =	Cord blood	N =	1 w – 2 m	N =	2 – 5 m	N =	5 – 9 m	N =	9 – 15 m	N =
B-lymphocytes[a]	136	10 (3-30)	18	14 (3-60)	11	17 (8-33)	12	16 (4-54)	13	15 (3-77)	10
NK-cells[a]	136	22 (8-62)	18	9 (3-23)	11	7 (2-20)	12	9 (2-36)	13	8 (1-64)	10
T-lymphocytes[a]	136	58 (38-88)	18	70 (55-90)	11	69 (49-97)	12	68 (49-95)	13	70 (56-87)	10
Helper T-lymphocytes[a]	136	41 (26-62)	18	52 (39-69)	11	50 (37-69)	12	46 (27-81)	13	46 (25-86)	10
Cytotoxic T-lymphocytes[a]	136	14 (5-37)	18	16 (7-35)	11	16 (6-41)	12	18 (10-35)	13	19 (7-58)	10
Naïve helper- cells[b]	125	80 (59-100)	18	86 (66-100)	10	88 (73-100)	11	86 (77-97)	11	86 (77-96)	10
Terminally differentiated helper T cells[b]	125	0.035 (0.0064-0.19)	18	0.017 (0.0053-0.052)	10	0.032 (0.004-0.25)	11	0.087 (0.00033-7.3)*	11	0.068 (0.00018-2.7)*	10
Effector memory helper T cells[b]	125	0.028 (0.0072-0.11)	18	0.052 (0.0025-1)	10	0.18 (0.057-0.59)	11	0.32 (0.07-1)	11	0.41 (0.0083-4)*	10
Central memory helper T cells[b]	125	17 (5-58)	18	13 (4-41)	10	10 (2-51)	11	11 (2-59)	11	12 (7-22)	10
Naïve cytotoxic T cells[c]	125	52 (6-100)	18	71 (44-100)	10	70 (47-100)	11	60 (31-100)	11	54 (16-100)	10
Terminally differentiated cytotoxic T cells[c]	125	14 (2-100)	18	11 (3-42)	10	10 (3-40)	11	19 (5-78)	11	20 (4-92)	10
Effector memory cytotoxic T cells[c]	125	6 (1-44)	18	5 (1-23)	10	6 (0.47-70)	11	9 (1-100)	11	12 (1-100)	10
Central memory cytotoxic T cells[c]	125	6 (0.22-100)	18	9 (2-35)	10	6 (0.08-24)*	11	3 (0.1-13)*	11	3 (2-6)	10
CD197 +/- cytotoxic T cells[c]	125	82 (66-100)	18	84 (66-100)	10	82 (63-100)	11	83 (62-100)	11	80 (49-100)	10
Recent thymic emigrants[b]	126	76 (61-94)	18	71 (50-100)	10	78 (64-94)	11	76 (65-90)	11	75 (61-93)	10
NK-T cells[a]	136	0.64 (0.12-3)	18	0.44 (0.13-1)	11	0.54 (0.23-1)	12	0.81 (0.17-4)	13	0.78 (0.14-4)	10
CXCR5$^+$ memory helper T cells[d]	102	3 (0.54-14)	14	6 (0.44-76)	10	15 (7-33)	11	20 (9-47)	10	22 (7-70)	10
Regulatory T cells[b]	124	7 (4-13)	18	8 (4-16)	10	8 (6-11)	11	8 (4-18)	11	8 (6-10)	10
TCR- alpha/beta[a]	127	51 (29-92)	18	66 (48-91)	10	63 (43-93)	11	60 (39-94)	11	63 (46-88)	10
Double negative T cells[e]	123	1 (0.38-3)	18	0.58 (0.24-1)	10	0.79 (0.18-3)	11	1 (0.39-4)	11	1 (0.42-2)	10
Helper T cells[e]	123	69 (53-91)	18	74 (55-99)	10	72 (58-89)	11	66 (48-90)	11	66 (38-100)	10
Cytotoxic T cells[e]	123	27 (14-49)	18	23 (10-50)	10	25 (14-43)	11	31 (15-61)	11	29 (12-74)	10
Double positive T cells[e]	123	1 (0.46-4)	18	1 (0.37-6)	10	1 (0.31-6)	11	0.9 (0.6-2.2)	11	0.67 (0.15-3)	10
TCR- gamma/delta[a]	127	2 (0.58-5)	18	2 (0.27-15)	10	3 (1-7)	11	3 (0.82-10)	11	3 (1-10)	10
Double negative T cells[f]	123	68 (54-85)	18	65 (42-100)	10	56 (37-83)	11	64 (36-100)	11	68 (54-85)	10
Helper T cells[f]	123	17 (7-39)	18	17 (3-91)	10	18 (9-33)	11	9 (4-24)	11	6 (0.35-100)	10
Cytotoxic T cells[f]	123	13 (5-35)	18	10 (0.96-100)	10	24 (15-37)	11	23 (8-67)	11	22 (8-58)	10
Double positive T cells[f]	123	0.52 (0.097-3)	18	0.71 (0.083-6)	10	1 (0.12-13)	11	0.58 (0.12-3)	11	0.28 (0.028-3)	10

w = week, m = month, y = year. [a] %of total peripheral lymphocyte population, [b] % of CD4$^+$ T-lymphocytes, [c] % of CD8$^+$ T-lymphocytes, [d] % of CD4$^+$CD45RO$^+$ T-lymphocytes, [e] % of TCR alpha/beta$^+$ T-lymphocytes, [f] % of TCR gamma/delta$^+$ T-lymphocytes. * The right limit of this tolerance interval was based on the original values (see Method). ** Age range: 16 – 76 years. Median age: 33 years.

Population	15 – 24 m	N =	2 -5 y	N =	5 – 10 y	N =	10- 16 y	N =	> 16 y**	N =
B-lymphocytes[a]	19 (8-45)	10	18 (8-39)	11	12 (4-33)	15	13 (7-24)	15	10 (4-28)	21
NK-cells[a]	11 (1-96)	10	6 (2-25)	11	8 (2-31)	15	14 (4-51)	15	15 (5-49)	21
T-lymphocytes[a]	60 (36-100)	10	69 (52-92)	11	73 (55-97)	15	68 (52-90)	15	67 (50-91)	21
Helper T-lymphocytes[a]	39 (16-91)	10	40 (25-66)	11	40 (26-61)	15	36 (20-65)	15	42 (28-64)	21
Cytotoxic T-lymphocytes[a]	16 (7-40)	10	21 (9-49)	11	25 (13-47)	15	24 (14-40)	15	22 (12-40)	21
Naïve helper- cells[b]	79 (56-100)	10	69 (52-92)	11	67 (46-99)	13	60 (37-97)	15	46 (16-100)	16
Terminally differentiated helper T cells[b]	0.095 (0.00032-4.1)*	10	0.14 (0.0062-1.2)*	11	0.1 (0.0031-1.8)*	13	0.17 (0.0042-5.8)*	15	0.35 (0.0083-6.8)*	16
Effector memory helper T cells[b]	0.67 (0.024-4.7)*	10	2 (0.26-9)	11	2 (0.27-18)	13	3 (0.49-25)	15	5 (1-23)	16
Central memory helper T cells[b]	10 (0.09-40)*	10	28 (15-56)*	11	18 (0.35-100)	13	32 (13-76)	15	42 (18-95)	16
Naïve cytotoxic T cells[c]	46 (10-100)	10	46 (19-100)	11	42 (16-100)	13	44 (20-95)	15	29 (6-100)	16
Terminally differentiated cytotoxic T- cells[c]	24 (8-71)	10	22 (6-83)	11	25 (15-41)	13	24 (9-65)	15	19 (7-53)	16
Effector memory cytotoxic T cells[c]	15 (2-100)	10	23 (10-55)	11	24 (5-100)	13	22 (4-100)	15	36 (14-98)	16
Central memory cytotoxic T cells[c]	3 (1-8)	10	3 (1-9)	11	3 (1-6)	13	3 (0.42-18)	15	5 (1-20)	16
CD197 +/- cytotoxic T cells[c]	76 (38-100)	10	72 (53-99)	11	68 (39-100)	13	71 (51-97)	15	53 (26-100)	16
Recent thymic emigrants[b]	66 (40-100)	10	62 (37-100)	11	58 (41-81)	14	50 (31-81)	15	33 (7-100)	16
NK-T cells[a]	0.93 (0.16-6)	10	2 (0.66-7)	11	3 (0.49-14)	15	3 (0.64-15)	15	4 (1-18)	21
CXCR5$^+$ memory helper T cells[d]	20 (8-51)	10	21 (6-72)	9	24 (7-85)	8	18 (7-47)	10	17 (5-56)	10
Regulatory T cells[b]	9 (6-13)	10	7 (3-17)	11	8 (4-14)	15	9 (4-20)	12	8 (4-17)	16
TCR- alpha/beta[a]	53 (26-100)	10	59 (31-100)	11	64 (44-92)	15	60 (39-92)	15	59 (36-98)	16
Double negative T cells[e]	2 (0.55-4)	10	3 (1-7)	11	2 (0.77-6)	15	2 (0.54-6)	14	2 (0.57-5)	13
Helper T cells[e]	66 (44-99)	10	62 (47-81)	11	59 (47-75)	15	58 (39-87)	14	63 (46-87)	13
Cytotoxic T cells[e]	30 (14-66)	10	33 (21-53)	11	37 (25-54)	15	37 (20-68)	14	33 (19-59)	13
Double positive T cells[e]	0.65 (0.14-3)	10	0.73 (0.28-2)	11	0.53 (0.2-1)	15	0.62 (0.22-2)	14	0.87 (0.15-5)	13
TCR- gamma/delta[a]	4 (1-13)	10	6 (0.92-38)	11	7 (2-24)	15	6 (2-17)	15	3 (0.83-11)	16
Double negative T cells[f]	72 (50-100)	10	83 (67-100)	11	84 (68-100)	15	84 (71-98)	14	77 (51-100)	13
Helper T cells[f]	5 (0.62-40)	10	3 (0.71-12)	11	2 (0.3-10)	15	1 (0.23-9)	14	1 (0.25-7)	13
Cytotoxic T cells[f]	19 (5-66)	10	12 (2-58)	11	12 (4-37)	15	13 (3-63)	14	18 (4-81)	13
Double positive T cells[f]	0.26 (0.016-4)	10	0.16 (0.017-2)	11	0.13 (0.036-0.47)	15	0.13 (0.013-1)	14	0.26 (0.075-0.94)	13

References

1. Erkeller-Yuksel FM, Deneys V, Yuksel B et al. Age-related changes in human blood lymphocyte subpopulations. J Pediatr 1992; 120:216–22.
2. Comans-Bitter WM, de Groot R, van den Beemd R et al. Immunophenotyping of blood lymphocytes in childhood. Reference values for lymphocyte subpopulations. J Pediatr 1997;130:388–93.
3. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM et al. Longitudinal survey of lymphocyte subpopulations in the first year of life. Pediatr Res 2000;47:528–37.
4. Shearer WT, Rosenblatt HM, Gelman RS et al. Lymphocyte subsets in healthy children from birth through 18 years of age: the Pediatric AIDS Clinical Trials Group P1009 study. J Allergy Clin Immunol 2003;112:973–80.
5. Hamann D, Roos MT, van Lier RA. Faces and phases of human CD8 T cell development. Immunol Today 1999;20:177–80.
6. van Baarle D, Kostense S, van Oers MH, Hamann D, Miedema F. Failing immune control as a result of impaired CD8+ T cell maturation: CD27 might provide a clue. Trends Immunol 2002;23:586–91.
7. Appay V, Dunbar PR, Callan M et al. Memory CD8+ T cells vary in differentiation phenotype in different persistent virus infections. Nat Med 2002;8:379–85.
8. Opiela SJ, Koru-Sengul T, Adkins B. Murine neonatal recent thymic emigrants are phenotypically and functionally distinct from adult recent thymic emigrants. Blood 2009;113:5635–43.
9. Hendricks DW, Fink PJ. Recent thymic emigrants are biased against the T-helper type 1 and toward the T-helper type 2 effector lineage. Blood 2011;117:1239–49.
10. Sakaguchi S. Naturally arising CD4+ regulatory t cells for immunologic self-tolerance and negative control of immune responses. Annu Rev Immunol 2004;22:531–62.
11. Kim CH, Rott LS, Clark-Lewis I, Campbell DJ, Wu L, Butcher EC. Subspecialization of CXCR5+ T cells: B helper activity is focused in a germinal center-localized subset of CXCR5+ T cells. J Exp Med 2001;193:1373–81.
12. Notarangelo LD, Fischer A, Geha RS et al. Primary immunodeficiencies: 2009 update. J Allergy Clin Immunol 2009;124:1161–78.
13. van Gent R, van Tilburg CM, Nibbelke EE et al. Refined characterization and reference values of the pediatric T- and B cell compartments. Clin Immunol 2009;133:95–107.
14. Reimann KA, Chernoff M, Wilkening CL, Nickerson CE, Landay AL. Preservation of lymphocyte immunophenotype and proliferative responses in cryopreserved peripheral blood mononuclear cells from human immunodeficiency virus type 1-infected donors: implications for multicenter clinical trials. The ACTG Immunology Advanced Technology Laboratories. Clin Diagn Lab Immunol 2000;7:352–9.
15. Hokland P, Heron I. Analysis of the lymphocyte distribution during Isopaque-Ficoll isolation of mononuclear cells from human peripheral blood. J Immunol Methods 1980;32:31–9.
16. Saule P, Trauet J, Dutriez V, Lekeux V, Dessaint JP, Labalette M. Accumulation of memory T cells from childhood to old age: central and effector memory cells in CD4(+) versus effector memory and terminally differentiated memory cells in CD8(+) compartment. Mech Ageing Dev 2006;127:274–81.
17. Kraan J, Gratama JW, Keeney M, D'Hautcourt JL. Setting up and calibration of a flow cytometer for multicolor immunophenotyping. J Biol Regul Homeost Agents 2003;17:223–33.
18. Hanh GJ, Meeker WQ. Statistical Intervals: A Guide for Practitioners. Hoboken, NJ: John Wiley and Sons, Inc., 1991. ISBN: 10: 0471887692, 13: 978-0471887690.
19. Horvath D, Kayser C, Silva CA, Terreri MT, Hilario MO, Andrade LE. Decreased recent thymus emigrant number in rheumatoid factor-negative polyarticular juvenile idiopathic arthritis. Clin Exp Rheumatol 2010;28:348–53.
20. Vieira QF, Kayser C, Kallas EG, Andrade LE. Decreased recent thymus emigrant number is associated with disease activity in systemic lupus erythematosus. J Rheumatol 2008;35:1762–7.
21. Just HL, Deleuran M, Vestergaard C, Deleuran B, Thestrup-Pedersen K. T cell receptor excision circles (TREC) in CD4+ and CD8+ T cell subpopulations in atopic dermatitis and psoriasis show major differences in the emission of recent thymic emigrants. Acta Derm Venereol 2008;88:566–72.

22. Hofer J, Hofer S, Zlamy M et al. Elevated proportions of recent thymic emigrants in children and adolescents with type 1 diabetes. Rejuvenation Res 2009;12:311–20.
23. Sakaguchi S, Miyara M, Costantino CM, Hafler DA. FOXP3+ regulatory T cells in the human immune system. Nat Rev Immunol 2010;10:490–500.
24. Torgerson TR, Ochs HD. Immune dysregulation, polyendocrinopathy, enteropathy, X-linked: forkhead box protein 3 mutations and lack of regulatory T cells. J Allergy Clin Immunol 2007;120:744–50; quiz 51–2.
25. Horn J, Manguiat A, Berglund LJ et al. Decrease in phenotypic regulatory T cells in subsets of patients with common variable immunodeficiency. Clin Exp Immunol 2009;156:446–54.
26. Forster R, Mattis AE, Kremmer E, Wolf E, Brem G, Lipp M. A putative chemokine receptor, BLR1, directs B cell migration to defined lymphoid organs and specific anatomic compartments of the spleen. Cell 1996;87:1037–47.
27. Breitfeld D, Ohl L, Kremmer E et al. Follicular B helper T cells express CXC chemokine receptor 5, localize to B cell follicles, and support immunoglobulin production. J Exp Med 2000;192:1545– 52.
28. Schaerli P, Willimann K, Lang AB, Lipp M, Loetscher P, Moser B. CXC chemokine receptor 5 expression defines follicular homing T cells with B cell helper function. J Exp Med 2000;192:1553–62.

CHAPTER 3

Levels of Somatic Hypermutations in B Cell Receptors Increase during Childhood

Clin Exp Immunol. 2014 Nov;178(2):394-8.

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Abstract

Somatic hypermutation (SHM) is an important step in the antigen-driven B cell development creating B-lymphocytes expressing high-affinity antibody receptors. It is known that the peripheral B-lymphocyte compartments of healthy children and adults differ considerably. However, the development of SHM with age has not been studied in detail before. Therefore, we used the IgK-restriction enzyme hot-spot mutation assay (IgK-REHMA) to get an estimation of SHM levels in different age groups in order to relate this to the size of the memory B-lymphocyte subpopulations. We show that the level of SHM increases rapidly during the first two years of life. This reflects the changes of the memory B cell subpopulations, but also changes in the SHM within memory B cell subsets, probably reflecting an increase of secondary memory B cell responses.

Introduction

Somatic hypermutations (SHM) are introduced in a programmed process in the variable regions of immunoglobulin genes to generate higher-affinity B cell receptors (BCR) in memory B cells. In bone marrow, pro- and pre-B cells develop from hematopoietic stem cells, and a unique BCR is generated by V(D)J recombination of the immunoglobulin (Ig) heavy chain and light chain loci. Antigen exposure in the periphery induces activation and further differentiation into so-called switched memory B-lymphocytes (smB) in germinal center (GC) reactions in secondary lymphoid tissues by two separate processes: class-switch recombination (CSR), which leads to loss of surface IgM, and gain of surface IgG, IgA or IgE, and SHM, which leads to enhanced antibody affinity [1]. Non-switched memory B-lymphocytes (nsmB) mutate their immunoglobulin receptor at least partly outside of a germinal center, without CSR [2].
The peripheral B-lymphocyte compartments of healthy adults and children of various ages differ considerably [3]. It has not been studied in detail how SHM develops with increasing age, nor how it is related to the development of populations of smB and nsmB cells. We used the IgK-restriction enzyme hot-spot mutation assay (IgK-REHMA) [4] to get an estimation of SHM levels in healthy children of different age groups and in adults in order to relate this to the size of the B cell subsets. We compared these values to those obtained in a group of common variable immunodeficiency disorders (CVID) patients that we published before [5].

Material and methods

After approval by the medical ethics committees of the Jeroen Bosch Hospital and Erasmus MC, and after informed consent was obtained from the parents and patients >12 years, peripheral blood samples were collected from 7 neonates (cord blood), 49 healthy children (mean age 5.3 years, range 1 week – 15.6 years), 8 healthy adults (> 16 years) [3], and 23 CVID patients [5]. The blood was collected from healthy children and adults, who underwent venipuncture or blood sampling by heel prick or finger prick for other reasons (e.g. minor surgery). Patients with an active infection, suspected or proven diseases of the immune system, or on immunosuppressive therapy were excluded.
To estimate the frequency of SHM, the IgK-REHMA assay was used. In brief, a RT-PCR reaction was performed using a HEX-coupled Vk3-20U forward primer and a FAM-coupled Vk3-20L reverse primer. The PCR products were digested with *Dde*I and *Fnu4*HI and run on an ABI3130XL capillary sequencer (Applied Biosystems). Unmutated gene products could be visualized as 106- or 109-bp FAM-coupled fragments and mutated gene products as 244-bp FAM-coupled fragments [4]. This analysis estimates the amount of SHM in expressed Vk3/20 gene segments in a specific sequence motif (hotspot), displayed as percentage of mutated segments within the total peripheral blood cell population. Because only B-lymphocytes express these gene segments, this percentage

is a reflection of the percentage of mutated cells within the peripheral population of B-lymphocytes. However, the amount of SHM within a specific B cell subpopulation cannot be estimated.

Flowcytometric immunophenotyping with directly labeled monoclonal antibodies was used to determine the following lymphocyte subpopulations: B-lymphocytes ($CD19^+$), transitional B cells ($CD19^+CD38^{++}IgM^{++}$), naive B-lymphocytes ($CD19^+CD27^-IgM^+IgD^+$), nsmB comprising natural effector cells ($CD19^+CD27^+IgM^+IgD^+$) and IgM-only B-lymphocytes ($CD19^+CD27^+IgM^+IgD^-$), and smB ($CD19^+CD27^+IgM^-IgD^-$) [3, 5].

Statistical calculations were performed with SPSS 20 for Windows. We calculated the Pearson product-moment correlation between the estimated percentage of SHM and age (alpha = 0.05, two-tailed) with the logarithmic value of age in days, with cord blood designated as day 1, to cope with the non-linear, downward bending relationship between the estimated SHM percentage and age.

Results

We found that the percentage of mutated hot spots in rearranged Vk3-20 gene segments in peripheral blood using the IgkREHMA assay, which gives an estimate of the level of SHM, shows an increase during the first two years of life in healthy children (Figure 1a), and then remains at a relatively stable level throughout childhood (mean 68%, range 38-89%, n=40). The Pearson product-moment correlation between the estimated percentage of SHM and age was significant and high (r(64) = 0.848, p=0.000). This pattern is clearly different from the development of the relative as well as the absolute numbers of smB and nsmB with increasing age (Figure 2; described before in reference 3) [3].

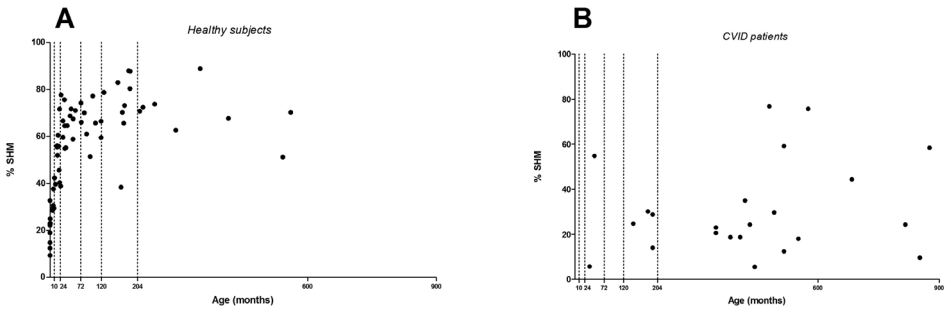

Figure 1
Percentage of somatic hypermutation (SHM) using the Igk-REHMA assay distributed for age. Every dot represents a healthy subject [**A**] or a CVID patient [**B**]. The dotted lines show the age groups used in the statistical analyses.

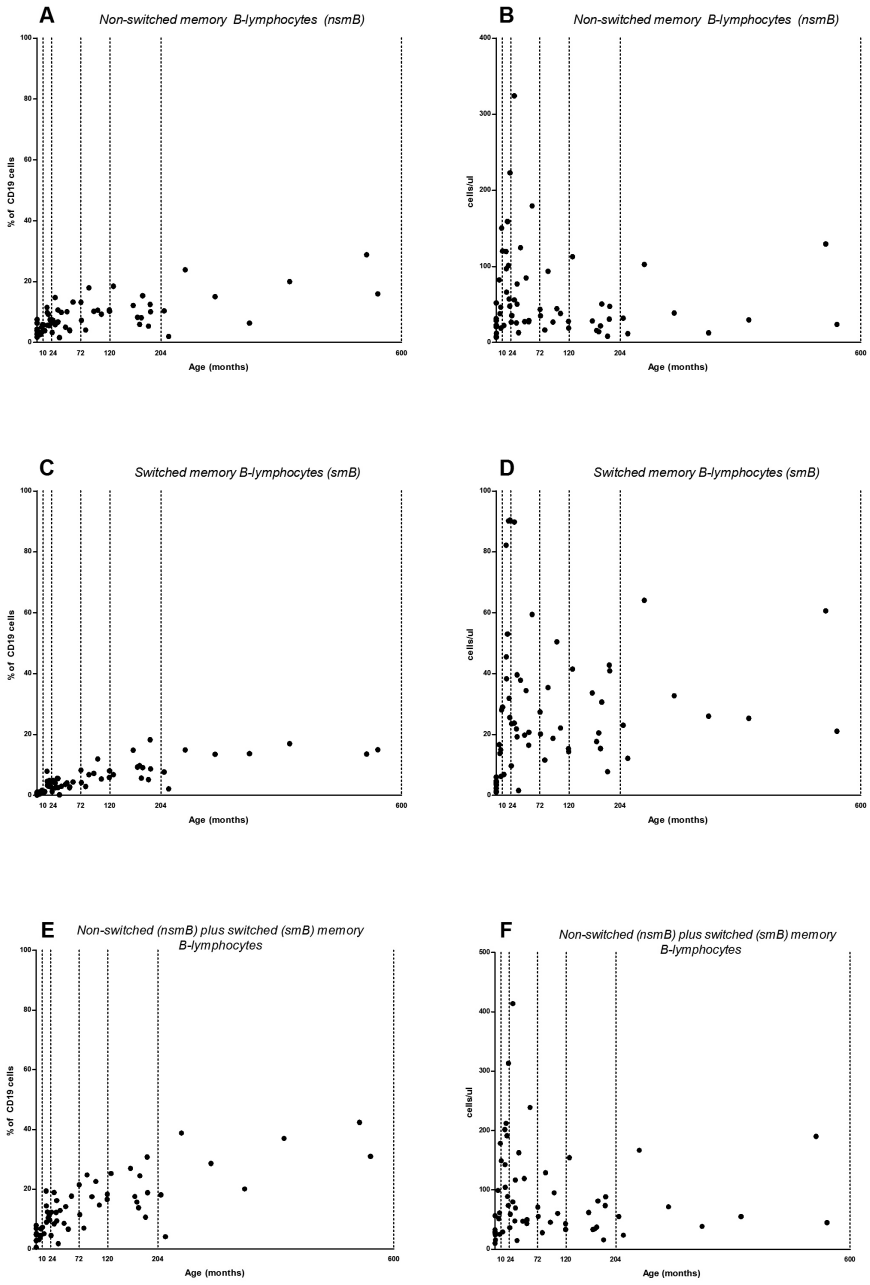

Figure 2
Percentages of non-switched memory B-lymphocytes (nsmB) [**A**], switched memory B-lymphocytes (smB) [**C**] and nsmB + smB [**E**] within the total peripheral B cell population ($CD19^+$) distributed for age. Absolute numbers of non-switched memory B-lymphocytes (nsmB) [**B**], switched memory B-lymphocytes (smB) [**D**] and nsmB + smB [**F**] distributed for age. Every dot represents a healthy subject. The dotted lines show the age groups used in the statistical analyses.

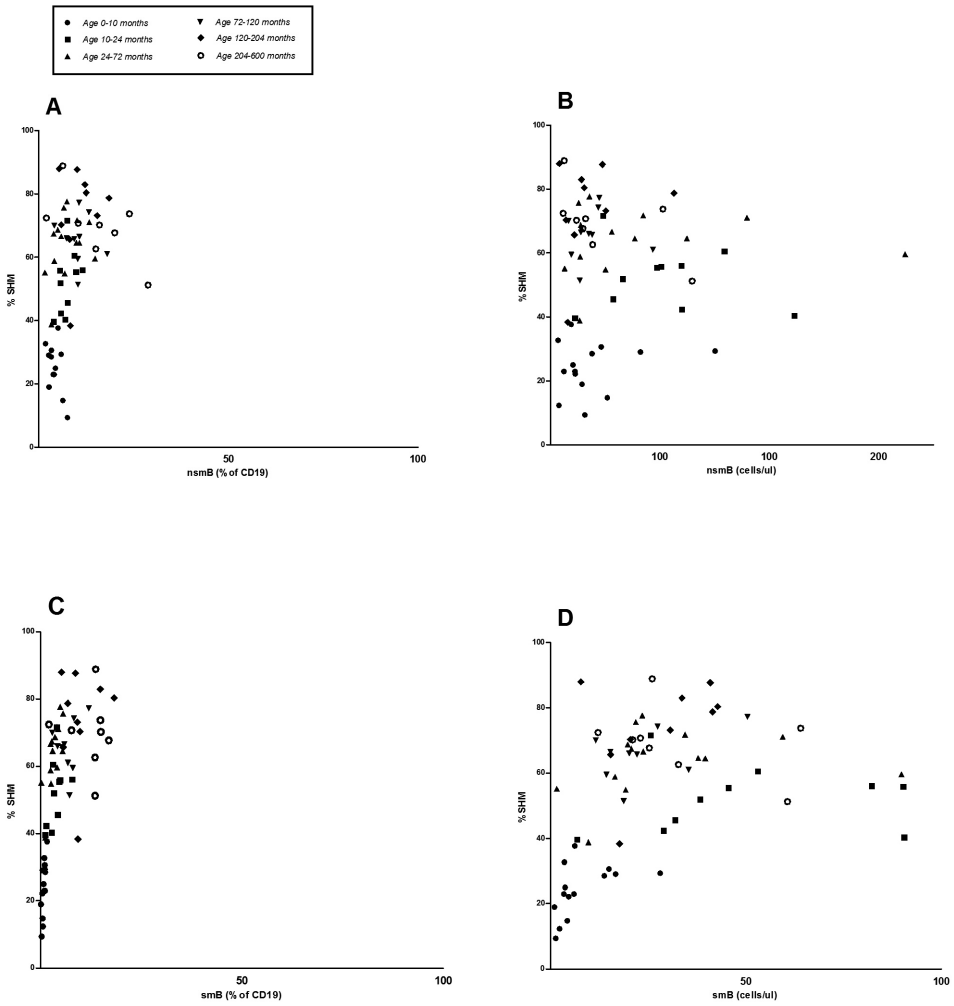

Figure 3
Percentage of somatic hypermutation (SHM) using the Igk-REHMA assay distributed for percentages [**A**] and absolute numbers [**B**] of non-switched memory B-lymphocytes (nsmB), and for percentages [**C**] and absolute numbers [**D**] of switched memory B-lymphocytes (smB). Every dot represents a healthy subject.

There were also significant correlations between the percentage of SHM and the absolute numbers ($r(64) = 0.354$, $p=0.004$) and percentages of smB ($r(64) = 0.624$, $p=0.001$), as well as the percentages ($r(62) = 0.395$, $p=0.002$) but not the absolute numbers of nsmB ($r(62) = -0.019$, $p=0.881$) (Figure 3). The strong correlation between age and the percentage of SHM is still evident after correction for the absolute numbers of smB (partial correlation estimated percentage of SHM and age = 0.804, $p=0.000$) and the percentages of smB (partial correlation = 0.713, $p=0.000$). The same applies to the

absolute numbers of nsmB (partial correlation estimated percentage of SHM and age = 0.810, p=0.000) and the percentages of nsmB (partial correlation = 0.767, p=0.000). So, SHM increases more than expected based on the memory B cell subset size alone, suggesting an increase in SHM frequency in single B cells.
As described before, CVID patients show variable, lower estimated percentages of SHM (mean percentage SHM 31%, range 6-77%, n=23; Figure 1b) compared to the healthy subjects (t[85]=4.917, p=0.000) [5].

Discussion

SHM is an important step in the antigen-driven B cell development creating B-lymphocytes expressing high-affinity antibody receptors. We show that the estimated level of SHM, using the IgK-REHMA assay, steeply increases during the first two years of life. This increase was associated with changes in size of the two B-lymphocyte subpopulations that contain SHM (smB and nsmB), but this could not explain the total observed increased SHM frequency. We hypothesize that the increase of SHM in childhood reflects an increase of the proportion of memory B cells originating from secondary GC-dependent B cell responses as well as an increase of the SHM frequency within the memory B cell compartment [6], reflecting immune maturation. A study in sorted (memory) B cells could confirm this hypothesis. However, this would need a lot more blood, which poses an ethical problem in young, otherwise healthy children.
Previous studies have shown that SHM levels are decreased in certain subgroups of CVID, but this was accompanied by diminished isotype switching, decreased B cell proliferation or decreased switched memory B cell subpopulations [4, 5, 7, 8]. So far, the clinical consequences of low levels of SHM by itself are unknown. A delayed maturation of SHM levels in the presence of normal memory B cell subset counts might therefore reflect the presence of persistent primary B cell responses and hence impaired immune maturation. We propose that if such a phenomenon exists, it may be associated with recurrent infections, which has to be explored in further studies.
In conclusion, this study shows the increase of estimated percentages of SHM as measured by the IgK-REHMA assay in the first two years of life. This reflects changes of the memory B cell subpopulations in young children, but also in the SHM frequency within memory B cell subsets, probably reflecting an increase of secondary memory B cell responses. Sequencing of large numbers of immunoglobulin genes at the B cell subset level in young children, e.g. by using next generation sequencing, should be performed in future studies to confirm this hypothesis.

Acknowledgments
We would like to thank Sandra Posthumus-van Sluijs from the Department Immunology of the Erasmus Medical Centre Rotterdam for performing the IgK-REHMA assays.

References

1. LeBien TW, Tedder TF. B-lymphocytes: how they develop and function. Blood. 2008;112(5):1570-80.
2. Weller S, Braun MC, Tan BK, Rosenwald A, Cordier C, Conley ME, et al. Human blood IgM "memory" B cells are circulating splenic marginal zone B cells harboring a prediversified immunoglobulin repertoire. Blood. 2004;104(12):3647-54.
3. Schatorje EJ, Gemen EF, Driessen GJ, Leuvenink J, van Hout RW, van der Burg M, et al. Age-matched reference values for B-lymphocyte subpopulations and CVID classifications in children. Scand J Immunol.74(5):502-10.
4. Andersen P, Permin H, Andersen V, Schejbel L, Garred P, Svejgaard A, et al. Deficiency of somatic hypermutation of the antibody light chain is associated with increased frequency of severe respiratory tract infection in common variable immunodeficiency. Blood. 2005;105(2):511-7.
5. Driessen GJ, van Zelm MC, van Hagen PM, Hartwig NG, Trip M, Warris A, et al. B cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood. 2011;118(26):6814-23.
6. Berkowska MA, Driessen GJ, Bikos V, Grosserichter-Wagener C, Stamatopoulos K, Cerutti A, et al. Human memory B cells originate from three distinct germinal center-dependent and -independent maturation pathways. Blood. 2011;118(8):2150-8.
7. Levy Y, Gupta N, Le Deist F, Garcia C, Fischer A, Weill JC, et al. Defect in IgV gene somatic hypermutation in common variable immuno-deficiency syndrome. Proc Natl Acad Sci U S A. 1998;95(22):13135-40.
8. Bonhomme D, Hammarstrom L, Webster D, Chapel H, Hermine O, Le Deist F, et al. Impaired antibody affinity maturation process characterizes a subset of patients with common variable immunodeficiency. J Immunol. 2000;165(8):4725-30.

PART TWO

Patient Registries and Surveys

What can we learn from them?

CHAPTER 4

The PedPAD Study: Boys predominate in the Hypogammaglobulinemia Registry of the ESID Online Database

Clin Exp Immunol. 2014 Jun;176(3):387-93.

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Abstract

Hypogammaglobulinemias are the most common primary immunodeficiency diseases. This group of diseases is very heterogeneous, and little is known about these diseases in children. In the Pediatric Predominantly Antibody Deficiencies (PedPAD) study, we analysed data from the ESID Online Database to get more insight into the characteristics of children with hypogammaglobulinemia; 46 centers in 18 different countries agreed to participate. Data from 2076 out of the 3191 children who were registered at the time of data extraction with a diagnosis of hypogammaglobulinemia (this excludes agammaglobulinemia and defects in class switch recombination) were available for analysis. The data set showed several limitations. Because of country related differences in diagnostic criteria used for classification of different types of primary hypogammaglobulinemia, further analysis of the data was performed in the combined data set. The most striking observation is the strong majority of male patients in the group of children with primary hypogammaglobulinemia (n=1292, 63%). This male predominance was observed in each of the 18 countries involved. The boys were younger at diagnosis (mean age males 5.3 years; mean age females 5.8 years). Moreover, one or more complications were more frequently reported in boys (12%) as compared to girls (5%). The male predominance suggests that patients with an undetected or unknown X-linked genetic cause are included in this group of children registered as primary hypogammaglobulinemia.

Introduction

Primary immunodeficiencies (PID) are inherited defects of the immune system, with predominantly antibody deficiencies (PAD) being the largest subgroup. The hallmark of PAD is a marked reduction or absence of immunoglobulins, with an increased susceptibility to mostly bacterial infections that typically involve the upper and lower respiratory tract. PADs can be divided into agammaglobulinemias, defects of class switch recombination and hypogammaglobulinemias. 'Hypogammaglobulinemia' is by far the most common entity, comprising nearly half of all PID diagnoses [1]. For most diseases in the first two subgroups, the genetic defects have been described, and their effects on B cell differentiation studied [2]. The hypogammaglobulinemia subgroup, however, is very heterogeneous, and much less is known about these diseases. Among the hypogammaglobulinemias, common variable immunodeficiency disorders (CVID) is the most commonly reported diagnosis in registries, followed by immunoglobulin (Ig) G subclass deficiency [1].

Because PIDs are relatively rare disorders, international collaboration is necessary to study the overall clinical characteristics of these diseases. Since 2004, the European Society for Immunodeficiencies (ESID) has been running an online database for primary immunodeficiencies: the ESID Online Database. This database registers demographical, clinical and laboratory characteristics of patients with PID [3]. At present, more than 18,000 cases from 119 centers in Europe are registered [4]. Biennial reports from the ESID Online Database have been published, but none of these reports has focussed specifically on children [1, 5, 6]. Also, in other available literature most published data on hypogammaglobulinemia refer to adult patients.

Children with hypogammaglobulinemia constitute a special group due to their maturing immune system. Some antibody deficiencies disappear over time and seem to be a physiological variation, whereas other antibody deficiencies remain and manifestations of more severe immunodeficiency will develop. Currently, it is impossible to predict which child will get better and which one will suffer from more or less severe disease in the future. Moreover, data from adults cannot be simply extrapolated to children. Separate studies have to be performed. We used the data from the ESID Online Database to get more insight in the characteristics of children with hypogammaglobulinemia.

Materials and Methods

The objective of this study was to describe numbers, geographical distribution, underlying diseases, age and laboratory values at presentation and diagnosis, the diagnostic delay and follow-up data in children with hypogammaglobulinemia (this excludes agammaglobulinemia and defects in class switch recombination) registered in the ESID Online Database.

The system and structure of the ESID Online Database has been described before [3, 5]. The data of the ESID Online Database are stored on secure servers of the IT Centre of the University Hospital in Freiburg, Germany. The categorization is based on the classification defined by the International Union of Immunological Societies (IUIS) [7]. There is a set of identical fields for all patients, which comprises diagnosis, gender, age of patient at diagnosis, information whether it is a sporadic or familial case, current medication, adverse effects of treatment, basic laboratory values, and genetic mutation data. These fields form the 'core data set'. Patients give informed consent before their data are entered into the database. The aim of the database is long-term documentation of a patient; documentation is requested at least once a year for each patient. The date of a patient's attendance at the clinic is recorded and data associated with this patient visit, such as a change in the treatment regimen can be documented. Some centres and national networks are maintaining local databases for their PID patients. Their data are transferred electronically to the ESID database at regular intervals. The database has an inbuilt automatic quality assurance system including field type, range and plausibility checks. In addition, data sets are checked regularly for plausibility and completeness by the database administration.

For this study, all centers that registered children with hypogammaglobulinemia (age <19 years) were approached; 46 centers from 18 countries agreed to participate. Only data from patients reported by these collaborating centers were included in our study.

Standard statistical analyses were applied to investigate associations between categorical variables (Chi square analysis and the Fisher exact test [Monte Carlo; 10,000 samples] when expected cell values were lower than 5) and differences between groups (the t-test [comparing two groups] and analysis of variance [comparing more than two groups]). That means that the data can be summarized as frequencies in cross-tabulations, e.g. by cross-tabulating country by gender, consanguinity cases (yes/no) or familial cases (yes/no). When age variables were involved (age of onset, presentation or diagnosis, all continuous variables) differences between groups were tested by using the t-test (comparing two groups) and analysis of variance (comparing more than two groups). The statistical software package used was IBM SPSS statistics 20.

Results

Data of 2076 out of 3191 children registered within the 'Hypogammaglobulinemias' registry at the time of data extraction were available for analysis. These data were collected in 46 centers in 18 different countries; some countries only reported one or a few diagnoses (Supplementary table 1). Estonia (20), Turkey (14), the Netherlands (8), Spain (4) and Greece (4) reported most affected children per one million inhabitants (Figure 1). In absolute numbers, Turkey reported most children (n=968), followed by Germany (n=257) and Spain (n=182).

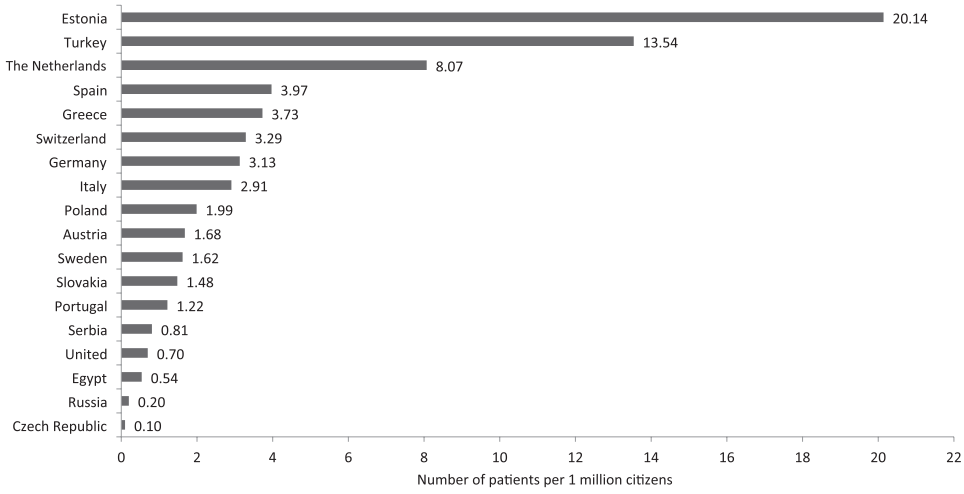

Figure 1
Number of pediatric patients per 1 million inhabitants registered in the ESID Online Database per country participating in the PedPAD study.

Unfortunately, the data set showed several limitations, making interpretation of the data challenging. Obvious mistakes were excluded from further analysis (e.g. date of diagnosis or laboratory value before date of birth or clinical presentation), as well as laboratory data without dates or units. Many entries were incomplete (Table 1); children frequencies in the statistical analyses may vary because of differences in data available in subsets of data. IgG-substitution was not clearly documented in most cases, therefore no analysis of reported IgG-levels was performed (IgG-substitution was reported for 1152 episodes in 598 patients; 813 intravenously, 331 subcutaneously, 2 intramuscularly administered, and 6 by unknown route).

Furthermore, when comparing immunoglobulin levels and diagnoses, we noticed that different criteria for registering under a certain diagnosis were used in different countries. The current ESID diagnostic criteria (www.esid.org) were not always followed. Because reliable immunoglobulin levels at diagnosis were lacking in many patients, diagnosis could not be checked in every patient [4]. Therefore, further analysis of the data was performed *within the total group* of children with *primary* hypogammaglobulinemia which includes the subregistries common variable immunodeficiency (CVID), deficiency of specific IgG, IgA with IgG subclass deficiency, isolated IgG subclass deficiency, other hypogammaglobulinemias, other immunoglobulin gene deletions, selective IgA deficiency, selective IgM deficiency and transient hypogammaglobulinemia of infancy (THI) of the registry (Table 2; n=2052).

Table 1. Available information in the 'Hypogammaglobulinemias' registry of the ESID Online Database.

Data	Number of patients with available data	Percentage of total group
Data on infections	414	20%
Data on auto-immunity	85	4%
Data on other concomitant diseases	276	13%
Data on therapy	1312	63%
Data on immunizations	16	0.8%
Data on blood count	1830	88%
Data on immunoglobulins	1872	90%
Data on IgG-subclasses	890	43%
Data on white blood cells	1333	64%
Data on T and B cells	827	40%
Data on additional lab values	319	15%
Total	**2076**	**na**

na = not applicable.

General characteristics

The mean age at onset (defined as first symptoms) was 2.6 years (SD 3.15, median 1.0 years, range 0-18 years, n=1833). The mean age at diagnosis was 5.5 years (SD 3.98, median 5.0 years, range 0-19 years, n=1508), resulting in a mean diagnostic delay of 2.6 years (SD 2.87, median 2.0 years, range 0-17 years, n=1508). No information about survival was available in 594 patients (30%), 1416 patients (69%) were reported as alive at the last update (October 9th 2012). Although there were statistically significant differences in age at onset and age at diagnosis between countries, these were rather small (effect sizes of less than 0.10). In the sample of 1508 patients for whom the relevant age data was present, six countries had a small sample of 10 patients or less. We therefore excluded the Czech Republic (n=1), Austria (n=3), Portugal (n=8), Serbia (n=5), Slovakia (n=2) and Sweden (n=9) from this analysis. An analysis of variance was applied, giving a significant effect for age at diagnosis ($F[1,11] = 2.217$, $p=0.012$, partial $\eta^2 = 0.016$) and age at presentation ($F[1,11] = 3.818$, $p=0.000$, partial $\eta^2 = 0.028$). Switzerland (5.17, n=18) had a mean age at presentation later than 4 years. Russia (7.4, n=18) had a mean age at diagnosis later than 7 years. No relevant subgroups were detected in a post-hoc analysis. Consanguinity was present in 133 patients (6%), absent in 1590 patients (78%), and not reported in 329 (16%). The consanguinity differences between the countries were significant (Fisher exact test = 120.68, df=17, p=0.000). Egypt (17 out of 37 patients, 47%) and Serbia (6 out of 6, 100%) reported by far the largest percentage of consanguineous cases, but several other countries showed higher percentages too: Turkey (87 out of 938 patients, 9%) Russia (3 out of 15 patients, 20%), Sweden (3 out of 14 patients, 21%) and Austria (1 out of 6 patients, 17%). Consanguinity was not associated with gender (χ^2 [1, n=1723] = 1.236, p=0.266), age at onset ($t[1409] = -1.056$, $p=0.291$) or age at diagnosis ($t[1409] = -1.079$, $p=0.281$).

Table 2. Different diagnoses reported in the 2076 children from the 'Hypogammaglobulinemias' registry.

Diagnosis	Number of patients reported	Percentage of total hypogamma-globulinemia group	Percentage of boys
Common variable immunodeficiency (CVID)	469	23%	64%
Deficiency of specific IgG	39	2%	56%
IgA with IgG subclass deficiency	12	0.6%	67%
Isolated IgG subclass deficiency	301	15%	59%
Other hypogammaglobulinemias	188	9%	61%
Other immunoglobulin gene deletions	1	0.1%	100%
Secondary hypogammaglobulinemia	8	0.4%	unknown*
Secondary selective IgA deficiency	15	0.7%	unknown*
Selective IgA deficiency	513	25%	58%
Selective IgM deficiency	41	2%	89%
Thymoma with immunodeficiency	1	0.1%	unknown*
Transient hypogammaglobulinemia of infancy	488	24%	69%
TOTAL HYPOGAMMAGLOBULINEMIA GROUP	**2076**	**100%**	**63%**

Only the diagnoses in italics were included in the group of "total *primary* hypogammaglobulinemia" that were used for further analysis. *Percentage cannot be calculated due to lack of data.

Familial cases were present in 198 patients (10%), absent in 1500 patients (73%), and not reported in 354 patients (17%). The frequencies of familial cases between the countries differed significantly (Fisher exact test = 99.93, p=0.000). High numbers (more than 20%) were reported by the Netherlands (13 out of 34, 26%), Serbia (6 out of 6, 100%), and United Kingdom (6 out of 24, 25%). Familial cases were not associated with gender (χ^2 [1, n=1698] = 0.008, p=0.928), age at onset (t[1380] = -1.520, p=0.129) or age at diagnosis (t[1380] = 0.483, p=0.630). There were 58 family cases (28 families) reported with available information of the family member. If we look at the 19 families in which the child which was presented secondly was diagnosed after the firstly presented child, there was a significant decrease in diagnostic delay for the second child (one sample t test, test value 0: t(18) =-3.144, p=0.006).

A total of 297 patients reported consanguinity and/or familial cases. There was no association with gender (χ^2 [1, N=1763] = 0.592, p=0.442), age at onset (t[1434] = 1.653, p=0.096) or age at diagnosis (t[1434] = 0.948, p = 0.344. Egypt (17 out of 36, 47%) and Serbia (6 out of 6, 100%) reported significantly higher numbers than other countries.

A gene mutation was reported in only 12 patients (n=7 Transmembrane activator and calcium modulator and cyclophilin ligand interactor [TACI], n=4 Cluster of differentiation [CD]19, n=1 11q23 mutation).

Male patients are in the majority (63%; χ^2 based on an equal distribution of males and females (χ^2 [1, n=2052] = 137.93, p=0.000). This male predominance was present in each of the 18 countries. There was no specific association between country and gender

(Fisher exact test, p=0.446). Furthermore, male patients tend to be younger at onset (mean age male patients 2.7 years; mean age female patients 3.1 years; t [1509] = 1.83, p=0.068) and differ significantly at diagnosis (mean age male patients 5.3 years; mean age female patients 5.8 years; t[1509] = 2.38, p=0.012).

Laboratory characteristics

IgA levels were available in 1469 patients; IgM levels were available in 1477 patients. We categorized them as low, normal or high for age [8]; 643 (44%), 796 (54%) and 30 (2%) patients showed low, normal and high IgA levels at presentation, and 107 (7%), 1313 (89%) and 57 (4%) patients showed low, normal and high IgM levels at presentation, respectively (Figure 2). IgA and IgM levels were not associated with gender (IgA: χ^2 [2, n=1469] = 0.354, p=0.831; IgM: χ^2 [2, n=1477] = 3.38, p=0.187) or consanguinity (IgA: χ^2 [2, n=1299] = 0.679, p=0.712; IgM: χ^2 [2, n=1304] =0.498, p=0.780). Country was associated with IgA levels (Fisher exact test [n=1469] = 206.10, p=0.000) and with IgM levels (Fisher exact test [n=1477] = 138.08, p=0.000). Low IgA levels were reported frequently (more than 70%) in children from the Czech Republic (1 out of 1 patient, 100%), Egypt (26 out of 34 patients, 77%), Estonia (21 out of 27, patients 78%), Greece (25 out of 35 patients, 72%), Poland (37 out of 50 patients, 74%), Russia (15 out of 21 patients, 71%), Slovakia (7 out of 7 patients, 100%), Spain (52 out of 57 patients, 91%) and Sweden (6 out of 8 patients, 75%). Low IgM levels were reported frequently (more than 15%) in the Czech Republic (1 out of 1 patient, 100%), Egypt (8 out of 34 patients, 24%), Germany (23 out of 27 patients, 18%), Russia (9 out of 22 patients, 41%), Slovakia (3 out of 7 patients, 43%) and the United Kingdom (8 out of 23 patients, 35%). The 57 patients with reported high IgM-levels were equally distributed among the countries.

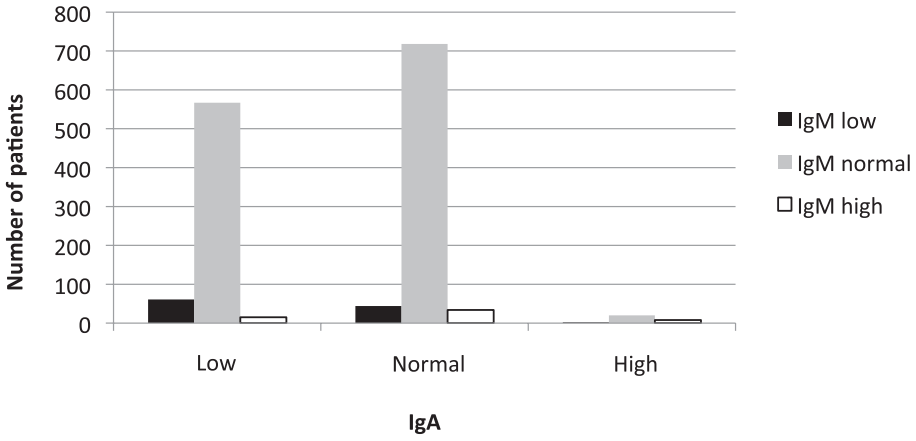

Figure 2
IgA and IgM levels at presentation of the children in the group of "total *primary* hypogammaglobulinemia". Patients are divided into different groups based on their levels of IgA and IgM at presentation: IgA low (left), IgA normal (middle), IgA high (right), IgM low (black), IgM normal (grey) and IgM high (white).

Blood counts (n=1247) showed 38 patients with severe neutropenia (<0.5 x10e9/L). Absolute numbers of B-lymphocytes (defined as $CD19^+$ lymphocytes) were reported in 780 patients, 23 without units. 52 of 757 patients (7%) show low absolute B cell numbers when compared to age matched reference values (range -0.004 to -0.18 x10e9/L below age matched reference value) [9]). For five patients absent B-lymphocytes were reported. Absolute T-lymphocyte numbers (defined as $CD3^+$ lymphocytes) were reported in 792 patients, 23 without units. 45 of 769 patients (6%) show low absolute T cell numbers when compared to age matched reference values (range -0.02 to -0.85 x10e9/L below age matched reference values) [9]. Absent T-lymphocytes were not reported.

Clinical phenotypes

We divided the hypogammaglobulinemia children with available data on complications (n=195 from 7 countries) in the different clinical phenotypes of CVID as described by Chapel in 2008 [10]: auto-immunity (n=80), polyclonal lymphocytic infiltration (n=31), enteropathy (n=7), and lymphoid malignancy (n=3). Additionally, the ESID Online Database contained two more relevant subgroups; 'granulomatous infiltration' (n=9) and 'pulmonary disease' (n=107). It was not possible to define patients 'without complications', because of the incompleteness of the data. Some patients (n=42) fitted several clinical phenotypes.

No differences between countries in clinical phenotypes were observed (Fisher exact test [n=237] = 35.99, p=0.720), nor between males and females (χ^2 [5, n=237] = 2.576, p=0.781). There was no association between clinical phenotype and gender (Fisher exact test [n=237] = 2.701, p=0.750). The clinical phenotypes were reported for 195 patients, 41 females (5% of the 760 females) and 154 males (12% of the 1292 males). Males turn out to be overrepresented in the clinical phenotypes, over and above their overrepresentation in the total sample (χ^2 [1, n=2052] = 23.688, p=0.000; odds ratio for gender = 2.373). This supports the hypothesis that male patients suffer more complications, and thus have more severe disease. However, there was no specific clinical phenotype that contained more boys.

Follow-up

Not all centers reported follow-up data for (all of) their patients; 1546 infectious episodes were reported in 413 patients. Czech Republic, Italy, Portugal, Serbia and the United Kingdom reported no follow-up data on infectious episodes. Most infections were localized in the respiratory tract: upper respiratory tract infection (n=156), pharyngitis (n=283), bronchitis (n=183), otitis media (n=150) and pneumonia (n=283). In addition, 1227 episodes of antimicrobial treatment were reported in 601 patients: antibiotics (n=1150), antifungals (n=28), antimalarials (n=1), antimycobacterials (n=12), antiparasitics (n=4) and antivirals (n=32).

A total of 132 episodes of auto-immune disease were reported in 80 patients. Most frequently reported auto-immune diseases were: thyroiditis (n=16), coeliac disease (n=11), idiopathic thrombocytopenic purpura (n=20), insulin dependent diabetes mellitus

(n=14) and hemolytic anemia (n=25). No differences between male and female patients were found in reported auto-immune disease (χ^2 [1, n=2052] = 2. 264, p=0.132). However, country was associated with the frequency of auto-immune disease (Fisher exact test [n= 2052] = 14.945, p=0.000). Auto-immune diseases were reported frequently (more than 10%) in Austria (2 out of 14, 14%), Estonia (6 out of 27, 27%), Greece (13 out of 32, 31%), Poland (9 out of 76, 12%), and Sweden (4 out of 15, 27%). Age was only available for 39 patients with auto-immune disease. Mean age at onset in patients with reported auto-immune disease was 2.0 years and mean age at diagnosis was 5.5 years. The diagnostic delay was longer (2.6 years) in patients with auto-immune disease (t[1506]= -2.707, p=0.007).

Three cases of lymphoma were reported during follow-up, one each in Germany, Turkey and Spain; these were all male patients. One case of acute lymphoblastic leukemia was reported in a Dutch girl, and one case of a benign neoplasm of bone and articular cartilage was reported in a Spanish girl. Other concomitant diseases were reported in 605 patients, ranging from possibly related issues such as hepatosplenomegaly and Down syndrome to unrelated issues like concussion and dizziness. Only 'asthma' was reported in more than 1% of the total group (n=76, 4%). It is not clear whether this is truly asthma, or a misdiagnosis of airway disease caused by the immunodeficiency.

Discussion

Hypogammaglobulinemia in childhood is the laboratory manifestation of a very heterogeneous group of diseases; solid criteria for the diagnosis are difficult to define. The objective of this study was to obtain insight in the general, clinical and laboratory characteristics of children with hypogammaglobulinemia registered in the ESID Online Database and to see whether different clinical phenotypes could be defined. Analysis of the data was performed in the combined data set, because of country related differences in diagnostic criteria used for classification of different types of primary hypogammaglobulinemia. Although this hampers comparison with separate diagnostic groups, the overall data are still very useful. No such large data set in children with hypogammaglobulinemia has been reported so far.

Strikingly, male patients are clearly in the majority (63%), whereas the diseases in the sub-registry 'Hypogammaglobulinemias' are not known to be inherited by the X-linked route. This male predominance was seen in every country. Furthermore, their younger age at onset and at diagnosis suggests more severe disease. This is supported by the occurrence of more complications in boys (12%) as compared to girls (5%). Although not discussed explicitly before, this predominance of males can be found in previously published papers [1, 10, 11]. In the study of Chapel et al. in which CVID patients are classified in different clinical phenotypes 58% of patients were males (mean age 49.4 years) [10]. Also, in the latest update from the ESID Registry Working Party 60.8% of all registered patients were male, and 64.6% in the group of 15 years or younger [1]. A recent

update from the German registry for PID also shows a higher percentage of boys: 57.2% male in the overall group and twice as many boys in children below 12 years of age [11]. They attributed this male predominance to known X-linked diseases such as X-linked agammaglobulinemia (XLA) and Wiskott-Aldrich Syndrome (WASP). This can be an explanation for the male predominance overall, but it cannot explain the higher percentage of boys in the group of children with primary hypogammaglobulinemia, and suggests that this group also contains patients with diseases inherited by the X-linked route. This can be due to either insufficient diagnostic procedures or atypical presentation (i.e. a known X-linked PID diagnosis has been missed in both cases), or to as yet unknown X-linked diseases that cause primary hypogammaglobulinemia. Little is known as yet about the genetic defects in primary hypogammaglobulinemias. Genetic defects in CVID patients have been discovered in the past years (e.g. Inducible costimulator [ICOS], TACI, BAFF-R, CD19-complex and CD20), but in more than 95% of CVID patients the genetic background is as yet unknown [2]. A search for known and unknown X-linked genetic defects in boys with primary hypogammaglobulinemia could be very interesting. Focusing on boys with early onset hypogammaglobulinemia and complications will probably give the highest yield. At least, they need to be evaluated for B-lymphocyte numbers and known X-linked disease like a mutation in the Bruton's agammaglobulinemia tyrosine kinase gene.

A recent study from Driessen et al. showed that patients with idiopathic primary hypogammaglobulinemia (IPH), defined as patients with hypogammaglobulinemia who do not fulfill the diagnostic criteria for CVID, suffered from the same infectious complications as CVID patients (i.e. respiratory infections, bronchiectasis) but did not show the non-infectious complications (auto-immune cytopenia, polyclonal lymphocytic proliferation and persistent enteropathy) [12], which have been linked to increased mortality [10, 13]. In our study, auto-immune disease, polyclonal lymphocytic proliferation and enteropathy were reported in a minority of the total group of hypogammaglobulinemias (4%). Our data are compatible with the above interpretation, demonstrating a subgroup of patients with early onset of immune dysregulation.

Unfortunately, the current ESID Online Database has several limitations making interpretation of the data challenging; many data had to be excluded, and the determination of different clinical or laboratory phenotypes was not possible. Currently, the ESID Registry Working Party is reorganizing the database. There will be three levels of documentation. Level 1 will consist of a baseline form at initial registration and a short yearly follow-up form; this level will offer good insight into the prevalence of PID. Level 2 will consist of a diagnosis form and a current visit form, which is specific for each IUIS category; this level will give additional information on individual diseases. Temporary level 3 forms can be used for collecting in-depth information in dedicated studies. Because the quality of the data is dependent on the users who enter the data, more complex automated checks based on diagnostic criteria, training sessions with a uniform protocol containing clear definitions of diseases and the meaning of data fields and increased local as well as central monitoring are being considered. National registries

benefitted from employing data entry clerks, especially in the reporting rate [11, 14]. With the new database, more research on children with hypogammaglobulinemia will be possible in the future; particularly the level 3 option will be suited for this purpose.
In conclusion, this study gives a first overview of the general characteristics of children with primary hypogammaglobulinemia as registered in the current ESID Online Database. Determining different clinical or laboratory phenotypes was not possible because of the limitations in the collected data. The male predominance in the group of children with hypogammaglobulinemias suggests X-linked diseases are 'hidden' in this group. This finding warrants further exploration.

Acknowledgements
This study was approved and supported by the ESID Registry Working Party (Chair Prof. Dr. S. Ehl). We thank all patients and their parents for their consent to be registered in the ESID Online Database.
And ofcourse we would like to thank the PedPAD consortium: Alsina L (Hospital Sant Joan de Deu, Barcelona, Spain), Baumann U (Paediatric Pneumology, Allergology and Neonatology, Hannover Medical School, Germany), Belohradsky BH (Dr v. Haunersches Kinderspital, Ludwig Maximilians University, Munich, Germany), Bienemann K (University Childrens Hospital, Dusseldorf, Germany), Boardman B (University of Manchester, Manchester, United Kingdom), Borte M (Children's Hospital, Municipal Hospital 'St Georg', Academic Teaching Hospital of the University of Leipzig, Germany), Bredius RG (Department of Pediatrics, Leiden University Medical Center, the Netherlands), Brodszki N (Lund University Hospital, Sweden), Caracseghi F (Vall d'Hebron University Hospital, Barcelona, Spain), Ciznar P (Comenius University Medical School, 1st Pediatric Department, Bratislava, Slovak Republic), de Vries E (Department of Pediatrics, Jeroen Bosch Hospital, 's-Hertogenbosch, the Netherlands), Driessen GJ (Department of Pediatric Infectious Disease and Immunology, Erasmus Medical Center, University Medical Center Rotterdam, The Netherlands), Dückers G (HELIOS Klinikum Krefeld, Germany), Duppenthaler A (Universitätsklinik für Kinderheilkunde, Inselspital Bern, Switzerland), Farmaki E (Pediatric Immunology Referral Centre, First Dept of Pediatrics, Ippokration Hospital, Aristotle University of Thessaloniki, Greece),Galal N (Cairo University Specialized Paediatric Hospital, Egypt), Gennery A (Institute of Cellular Medicine, Newcastle University, United Kingdom), Gonzalez-Granado LI (University Hospital 12 octubre. Dept of Pediatrics, Madrid, Spain), Hlavackova E (Masaryk University, St. Anne University Hospital, Brno, Czech Republic), Hoernes M (University Children's Hospital Zürich, Childrens Research Center, Divison of Immunology, Switzerland), Kilic SS (Bursa, Uludag University Medical Faculty, Turkey), Krüger R (Berlin Charité, Germany), Kuijpers TW (Emma Children's Hospital, Academic Medical Center (AMC), Amsterdam, the Netherlands), Kütükcüler N (Ege University, Faculty of Medicine, Dept of Pediatric Immunology, Bornova, Izmir,Turkey), Llobet P (Hospital General, Granollers, Spain), Marques L (Hospital de Criancas Maria Pia, Porto, Portugal), van Montfrans JM (Wilhelmina Children's Hospital/University Medical Center Utrecht, Utrecht, The Netherlands), Papadopoulou-Alataki E (Aristotle University

of Thessaloniki, Medical School, Fourth Department of Pediatrics, Papageorgiou Hospital, Thessaloniki, Greece), Paschenko O (Russian Children's Clinical Hospital, Moscow), Pasic S (Mother & Child Health Institute, University of Belgrade, Serbia), Pietrogrande MC (for The Italian Primary Immunodeficiencies Network), Pignata C (Department of Translational Medical Sciences, "Federico II" University, Naples, Italy), Reda SM (Department of Pediatric Allergy and Immunology, Children's Hospital, Faculty of Medicine, Ain Shams University, Cairo, Egypt), Reisli I (Necmettin Erbakan University, Konya, Turkey), Roesler J (University Hospital Carl Gustav Carus, Dept. of Pediatrics, Dresden, Germany), Santos JL (Hospital Virgen de las Nieves, Granada, Spain), Schölvinck EH (Departments of Pediatrics, University Medical Centre Groningen, Groningen, The Netherlands), Schulze Ilka (Centre of Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Germany), Seidel MG (Division of Pediatric Hematology-Oncology, Department of Pediatrics and Adolescent Medicine, Medical University Graz, Austria), Shcherbina A (Research and Clinical Center for Pediatric Hematology, Oncology, Immunology, Moscow, Russia), Sundin M (Astrid Lindgren Children's Hospital and Karolinska Institutet, Sweden), Szaflarska A (Department of Clinical Immunology, Polish-American Institute of Paediatrics, Medical College, Jagiellonian University, Cracow, Poland), Velbri S (Tallinn Childrens' Hospital, Estonia), Warnatz K (Centre of Chronic Immunodeficiency (CCI), University Medical Center Freiburg and University of Freiburg, Germany), Warris A (Department of Pediatric Infectious Diseases and Immunology, Radboud University Nijmegen Medical Center, The Netherlands).

References

1. Gathmann B, Grimbacher B, Beaute J, Dudoit Y, Mahlaoui N, Fischer A, et al. The European internet-based patient and research database for primary immunodeficiencies: results 2006-2008. Clin Exp Immunol. 2009;157 Suppl 1:3-11.
2. van der Burg M, van Zelm MC, Driessen GJ, van Dongen JJ. New frontiers of primary antibody deficiencies. Cell Mol Life Sci. 2012;69(1):59-73.
3. Guzman D, Veit D, Knerr V, Kindle G, Gathmann B, Eades-Perner AM, et al. The ESID Online Database network. Bioinformatics. 2007;23(5):654-5.
4. Davies EG. Immunodeficiency in DiGeorge Syndrome and Options for Treating Cases with Complete Athymia. Front Immunol. 2013;4(2013):322.
5. Eades-Perner AM, Gathmann B, Knerr V, Guzman D, Veit D, Kindle G, et al. The European internet-based patient and research database for primary immunodeficiencies: results 2004-06. Clin Exp Immunol. 2007;147(2):306-12.
6. Gathmann B, Binder N, Ehl S, Kindle G. The European internet-based patient and research database for primary immunodeficiencies: update 2011. Clin Exp Immunol. 2012;167(3):479-91.
7. Al-Herz W, Bousfiha A, Casanova JL, Chapel H, Conley ME, Cunningham-Rundles C, et al. Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. Front Immunol. 2011;2:54.
8. de Vries E. Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol. 2012;167(1):108-19.
9. Schatorje EJ, Gemen EF, Driessen GJ, Leuvenink J, van Hout RW, van der Burg M, et al. Age-matched reference values for B-lymphocyte subpopulations and CVID classifications in children. Scand J Immunol.74(5):502-10.
10. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, et al. Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood. 2008;112(2):277-86.
11. Gathmann B, Goldacker S, Klima M, Belohradsky BH, Notheis G, Ehl S, et al. The German national registry for primary immunodeficiencies (PID). Clin Exp Immunol. 2013;173(2):372-80.
12. Driessen GJ, Dalm VA, van Hagen PM, Grashoff HA, Hartwig NG, van Rossum AM, et al. Common variable immunodeficiency and idiopathic primary hypogammaglobulinemia: two different conditions within the same disease spectrum. Haematologica. 2013;98(10):1617-23.
13. Chapel H, Lucas M, Patel S, Lee M, Cunningham-Rundles C, Resnick E, et al. Confirmation and improvement of criteria for clinical phenotyping in common variable immunodeficiency disorders in replicate cohorts. J Allergy Clin Immunol. 2012;130(5):1197-8 e9.
14. CEREDIH. The French national registry of primary immunodeficiency diseases. Clin Immunol. 2010;135(2):264-72.

Supplementary table 1. Number of reported patients for each diagnosis in the different countries in the ESID Online Database.

	AT	CH	CZ	DE	EE	EG	ES	GB	GR	IT	NL	PL	PT	RS	RU	SK	SE	TR
Common variable immuno deficiency (CVID)	5	2	1	110	1	25	50	23	10	65	59	33	1	6	16	7	1	54
Deficiency of specific IgG	0	1	0	21	0	0	2	2	0	0	11	0	0	0	0	0	0	2
IgA with IgG subclass deficiency	0	0	0	3	6	0	1	0	2	0	0	0	0	0	0	0	0	0
Isolated IgG subclass deficiency	7	2	0	41	4	0	4	2	6	0	14	4	0	0	3	0	1	213
Other hypogammaglobulinemias	1	19	0	45	1	0	2	7	0	0	35	0	0	0	1	0	7	70
Other immunoglobulin gene deletions	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Secondary hypogammaglobulinemia	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	1	3
Secondary selective IgA deficiency	0	0	0	4	0	0	0	0	0	0	0	0	0	0	0	0	0	11
Selective IgA deficiency	1	1	0	23	12	12	117	4	21	0	4	33	11	0	8	0	3	263
Selective IgM deficiency	0	0	0	4	0	0	4	1	0	0	3	0	0	0	0	0	0	29
Thymoma with immuno deficiency	0	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0
Transient hypogammaglobulinemia of infancy	0	0	0	9	3	2	2	4	3	110	7	6	1	0	0	1	3	337

AT = Austria, CH = Switzerland, CZ = Czech Republic, DE = Germany, EE = Estonia, EG = Egypt, ES = Spain, GB = United Kingdom, GR = Greece, IT = Italy, NL = the Netherlands, PL = Poland, PT = Portugal, RS = Serbian Republic, RU = Russian Federation, SK = Slovakia, SE = Sweden, TR = Turkey. Only the diagnoses in italics were included in the group of "total *primary* hypogammaglobulinemia" that were used for further analysis.

CHAPTER 5

The Challenge of Immunoglobulin-G Subclass deficiency and Specific Polysaccharide Antibody Deficiency

A Dutch Pediatric Cohort Study

J Clin Immunol. 2016 Feb;36(2):141-8.

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Abstract

Purpose

Immunoglobulin(Ig)G-subclass deficiency and specific polysaccharide antibody deficiency (SPAD) are among the most frequent causes of recurrent respiratory infections in children. Little is known about their prevalence, clinical presentation and prognosis. No study has been published in a Western-European nor in a mainly non-tertiary cohort until now. Therefore, we performed this observational cohort study in children recruited from secondary and tertiary pediatric practices all over the Netherlands.

Methods

Dutch pediatricians were monthly asked to report patients with IgG-subclass deficiency and/or SPAD. Demographic, clinical and laboratory characteristics were collected. Separate informed consent was asked from parents and children (≥12 years of age) for annual update of the medical status.

Results

49 children with confirmed IgG-subclass deficiency and/or SPAD were included. The majority of children (69%) was reported by four (out of 12) secondary hospitals with a pediatric immunologist in the staff. 45 children had ≥1 low IgG-subclass level and 11 had SPAD. IgG2 deficiency was the most prevalent IgG-subclass deficiency (37/49; 76%). 10% of these children already showed bronchiectasis. Two-thirds were male (33/49; 67%, p=0.015). From ten years of age, only boys were left and only boys showed progressive immunodeficiency during follow-up (11/24; 46%).

Conclusions

This is the first Western-European mainly non-tertiary cohort of children with IgG-subclass deficiency and/or SPAD. The disease course is not always benign, especially in boys. Most children were reported and managed in secondary hospitals with a pediatric immunologist in the staff. To identify more patients, the awareness of these diseases among general pediatricians should increase.

Introduction

Young children often suffer from respiratory infections, which are usually innocent and self-limiting. However, in some cases these infections are a sign of underlying primary immunodeficiency. It is difficult for pediatricians to decide in which children further investigations are warranted. Among primary immunodeficiencies, 'predominantly antibody deficiency' forms by far the largest subgroup [1]. Immunoglobulin (Ig) G-subclass deficiency and specific polysaccharide antibody deficiency (SPAD) are among the most common forms found in children who are analysed because of the clinical presentation of recurrent ear-nose-throat (ENT) and airway infections [2, 3].

IgG-subclass deficiency is defined as a deficiency in one or more IgG-subclasses (>2 SD below age-matched reference values) with normal or near normal IgG concentration. SPAD is diagnosed when there is profound alteration of the antibody response to polysaccharide antigens, either after documented invasive infection with e.g. S. pneumoniae or after test immunization with an unconjugated pneumococcal (or other) polysaccharide vaccine. For both diagnoses, T cell and more severe B cell defects should be excluded [4]. Children with IgG-subclass deficiency or SPAD can be asymptomatic, but if not typically present with increased susceptibility to bacterial ENT and respiratory tract infections, more often so when both IgG-subclass deficiency and SPAD are present in the same child [5]. Identifying children with IgG-subclass deficiency and/or SPAD among the many children seen in everyday practice can be challenging. Healthy young children can have up to 11 respiratory infections per year [6-8], so it is difficult to decide which children with recurrent infections are at the end of the normal spectrum and which children need further immunological work-up. Furthermore, it is not known which child with IgG-subclass deficiency and/or SPAD is just showing delayed maturation of the immune system with spontaneous resolve of the immunodeficiency within a few years, and in which child it is the first sign of a lasting primary antibody deficiency, or maybe even a development into a more severe one, like common variable immunodeficiency disorders (CVID).

Little is known about the prevalence, clinical presentation and prognosis of IgG-subclass deficiency and/or SPAD in children. An Australian study in a cohort from a tertiary center showed SPAD in 11/74 (15%) children with recurrent infections. SPAD was found in 10/91 (11%) children with recurrent infections in a Finnish cohort from a tertiary center [9, 10]. Both studies excluded children with concomitant other immunodeficiencies. SPAD was associated with allergic rhinitis and chronic otorrhea in these children, independent of age. In a retrospective study among 59 Turkish children with IgG-subclass deficiency from a tertiary center, IgG3-deficiency was most commonly found; 30% of these children reached normal values around the age of six years. In contrast, children with an isolated IgG2-deficiency in this cohort (9%) all remained IgG2-deficient during follow-up [11]. In a group of Thai children with recurrent infections recruited in a tertiary care hospital, 7/55 (13%) had an IgG-subclass deficiency. The most common presenting symptom was recurrent sinusitis (84%). Again, IgG3-deficiency was the most common IgG-subclass

deficiency found (56%) [12]. All of these studies were carried out in tertiary center cohorts and therefore in a highly selective population of children. No study has been published in a Western-European cohort until now, nor have children followed outside tertiary centers been included. Therefore, we performed this observational cohort study for IgG-subclass deficiency and/or SPAD in children recruited from secondary and tertiary pediatric practices all over the Netherlands, using the Netherlands Pediatric Surveillance Unit system to identify cases. More than one third of these children were only seen by a general pediatrician.

Methods

The objective of this study was to describe the number, geographical distribution, age, gender, clinical and laboratory characteristics of Dutch children diagnosed with IgG-subclass deficiency and/or SPAD, reported to the Netherlands Pediatric Surveillance Unit ("Nederlands Signalerings Centrum voor Kindergeneeskunde" [NSCK]). The NSCK monthly asks all pediatricians in the Netherlands to report certain selected cases which they have seen during that past month in their hospital (in- as well as outpatient clinic). Between March 1, 2009, and November 1, 2011, all Dutch pediatricians were monthly asked to report all children they had seen with both newly and previously diagnosed IgG-subclass deficiency and/or SPAD (case definition in Table 1).
Demographic, clinical and laboratory characteristics were collected from all reported patients and stored in a database using Research Manager® software developed by Cloud9 Health Solutions. Patient data were encrypted and saved on a protected server; these data did not contain information that enabled identification of the identity of the

Table 1. Case definition

1. Patient ≥2 and <18 years old, and
2. Serum IgG ≥4.0 g/l, and
3. a. One or more IgG-subclasses (IgG1, IgG2, and/or IgG3) below age-related normal[1] and/or
   b. If patient is ≥3 years old: inadequate response to 23-valent unconjugated pneumococcal vaccine (Pneumo23® from Aventis Pasteur MSD) measured by antibody titers before and 3-4 weeks after vaccination.[2]

N.B. all children born after the 1st of April 2008 received routine vaccination with 7-valent pneumococcal conjugate vaccine at the age of 2, 3, 4 and 11 months (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F). This means that the diagnosis of SPAD in them can only be based on the 16 other serotypes present in the 23-valent polysaccharide vaccine (1, 2, 3, 5, 7F, 8, 9N, 10A, 11A, 12F, 15B, 17F, 19A, 20, 22F, 33F).

[1] *de Vries E. Clin Exp Immunol 2012;167(1):108-19.*

[2] *Vaccination response was considered inadequate if:*
Antipneumococcal-IgG <20 U/ml and/or ≤4-fold increase of titer for type 3, and/or type 4 and 9
Or
≤4-fold increase of titer in at least 2 out of 4 of the evaluated serotypes (5, 7F, 8, 10A, 11A, 12F, 15B, 17F, 20, 33F) and one titer <0,35 µg/ml.

patients. Separate informed consent was asked from parents and children (≥12 years of age) for annual update of the medical status. The Medical Ethical Committee Brabant approved the study.
For the interpretation of the Ig levels, age-matched reference values were used [13]. Diagnosis of SPAD was made using the criteria displayed in Table 1. In the Netherlands, pneumococcal antibodies are only determined in certain laboratories, that use their own reference values. Until 2009, only three serotypes were determined using ELISA (conform an international standard protocol, referred to as the WHO protocol [14]), including the strongly-intermediate immunogenic serotypes 3, 4 and 9 [15, 16]. Since 2009, pneumococcal antibodies are determined using the Luminex system [17, 18] (initially 10 serotypes; 7 serotypes since 2014). In our cohort, 10 patients were diagnosed using the old method (3 serotypes) and 11 using the new method (10 or 7 serotypes).
One-sample tests (Chi-Square, Kolmogorov-Smirnov) were carried out with SPSS, version 21, to test age and gender distributions.

Results

In total, 116 potential study participants were reported to the NSCK by pediatricians from 36 different hospitals. After analysis of the data quality and completeness, 49 children with confirmed IgG-subclass deficiency and/or SPAD were included in the study (Figure 1). In this group, 45 children had ≥1 low IgG-subclass level and 11 children had SPAD (7 SPAD children had both IgG-subclass deficiency and SPAD, 1 child had SPAD and low-normal total IgG, and 3 children had only SPAD). Fourteen children had more than one IgG-subclass deficiency; IgG2 deficiency was most prevalent (Figure 2). Five children also had IgA deficiency, 5 other children had slightly decreased IgG levels and 4 children had additional IgA deficiency as well as slightly decreased IgG levels. None of the children had additional IgM deficiency (Figure 3).
The median age of the 49 children was 5.6 years (mean age 7.1 years, range 2-18 years). Two-thirds of these children were male (33/49; 67%, One-Sample Chi-Square test, p=0.015). From ten years of age, only boys were left (Figure 4). The age distribution for girls between 2 and 9 years of age is not uniform (One-Sample Kolmogorov-Smirnov test, p=0.05). The same applies to boys (p=0.039). This seems to suggest an age effect with a decreasing number of children in the age range between 2 and 9 years of age, but more data are needed to corroborate such an effect.
The majority (n=34; 69%) of these 49 children was reported from a secondary hospital with a pediatric immunologist in the staff (Figure 1). The clinical problems the children encountered in the last year before entering the study are shown in Table 2. A striking ten percent of children (4 boys, 1 girl) already showed bronchiectasis. In 32 children (63%) chronic use of medication was reported, mostly asthma medication (n=20) or prophylactic antibiotics (n=19). Four children received immunoglobulin substitution.
Follow-up data of 24 children were available with a median follow-up period of 40

months (range 12-65 months): 19 patients with IgG-subclass deficiency and 5 patients with IgG-subclass deficiency and SPAD. Nineteen children (79%) were followed in a secondary hospital with a pediatric immunologist in the staff (Figure 1). Six children (25%) did not suffer from recurrent infections anymore, and in 3 of them the IgG-subclasses had reached normal values for age (the other 3 children were not re-tested).

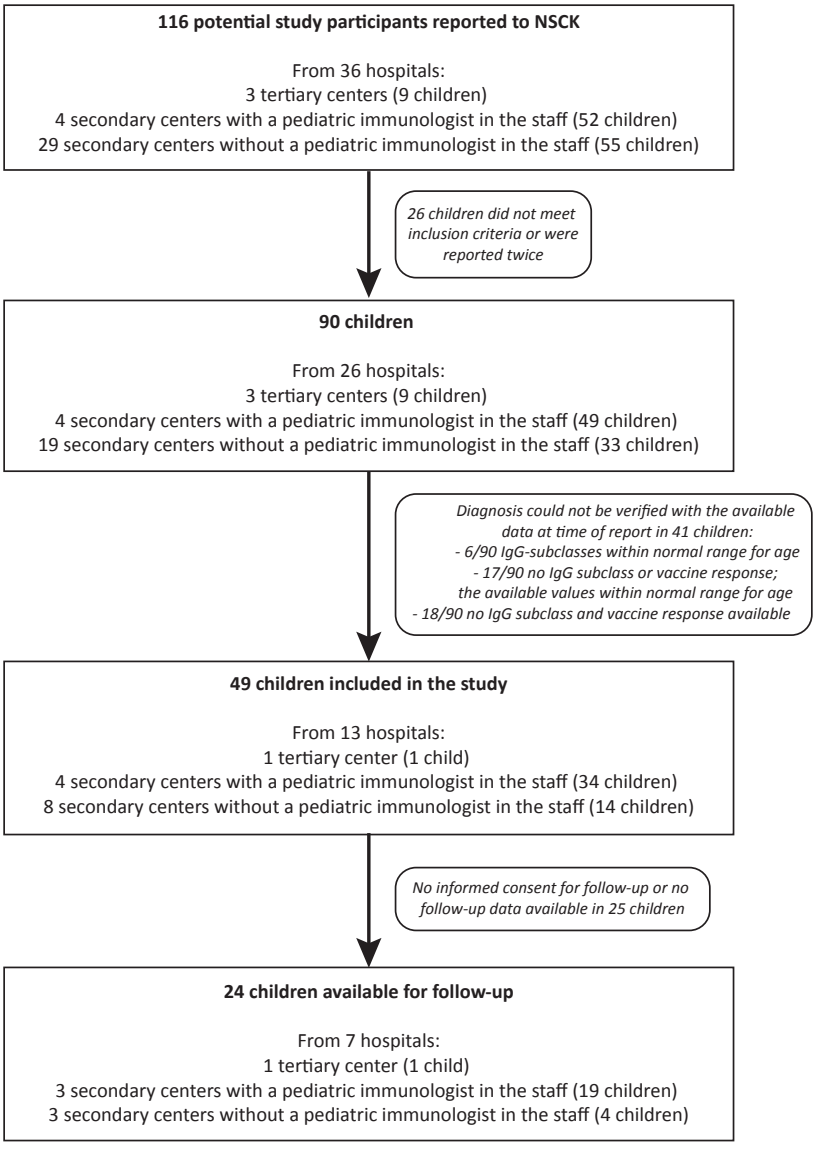

Figure 1
Study flowchart

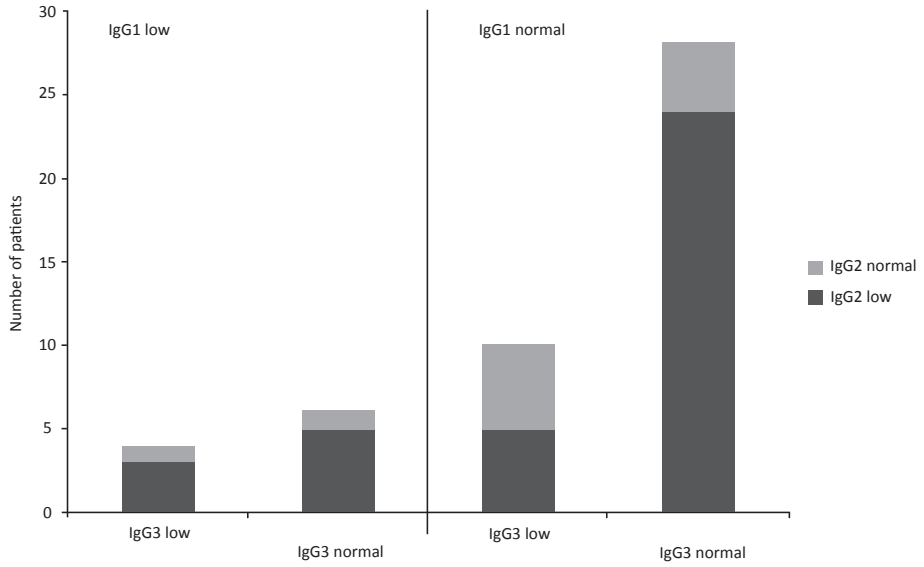

Figure 2.
Combination of IgG-subclass deficiencies

Table 2. Clinical problems in the past year at baseline

	Yes	No	Unknown
≥4 ENT infections*	36 (73%)	10 (20%)	3 (7%)
>1 lower airway infection	28 (57%)	19 (39%)	2 (4%)
Bronchiectasis	5 (10%)	26 (53%)	18 (37%)
Hearing loss	1 (2%)	5 (10%)	43 (88%)
Severe infections**	9 (18%)	25 (51%)	15 (31%)

* ENT = ear, nose and throat

** Severe infections *according to the reporting pediatrician*. These included pyelonephritis (n=2), urinary tract infection (n=2), adenoiditis (n=2), enterovirus meningitis (n=1), sepsis (n=1), H. pylori infection (n=1), and mesenterial lymphadenitis (n=1).

Seven children (29%) showed a similar clinical picture as at baseline, their IgG-subclass deficiency and/or SPAD had persisted, but had not worsened either. Progression of the immunodeficiency was reported in 11 children (46%), these were all boys: 1 boy developed decreased IgA levels, 3 boys developed decreased IgG levels, 3 boys developed decreased IgA and IgG levels, and 4 boys developed full-blown CVID. Eight children (33%; all of them boys) received immunoglobulin substitution therapy at follow-up: 4 of them had initially presented with IgG-subclass deficiency combined with SPAD, 3 of them already had immunoglobulin substitution at the time of reporting.

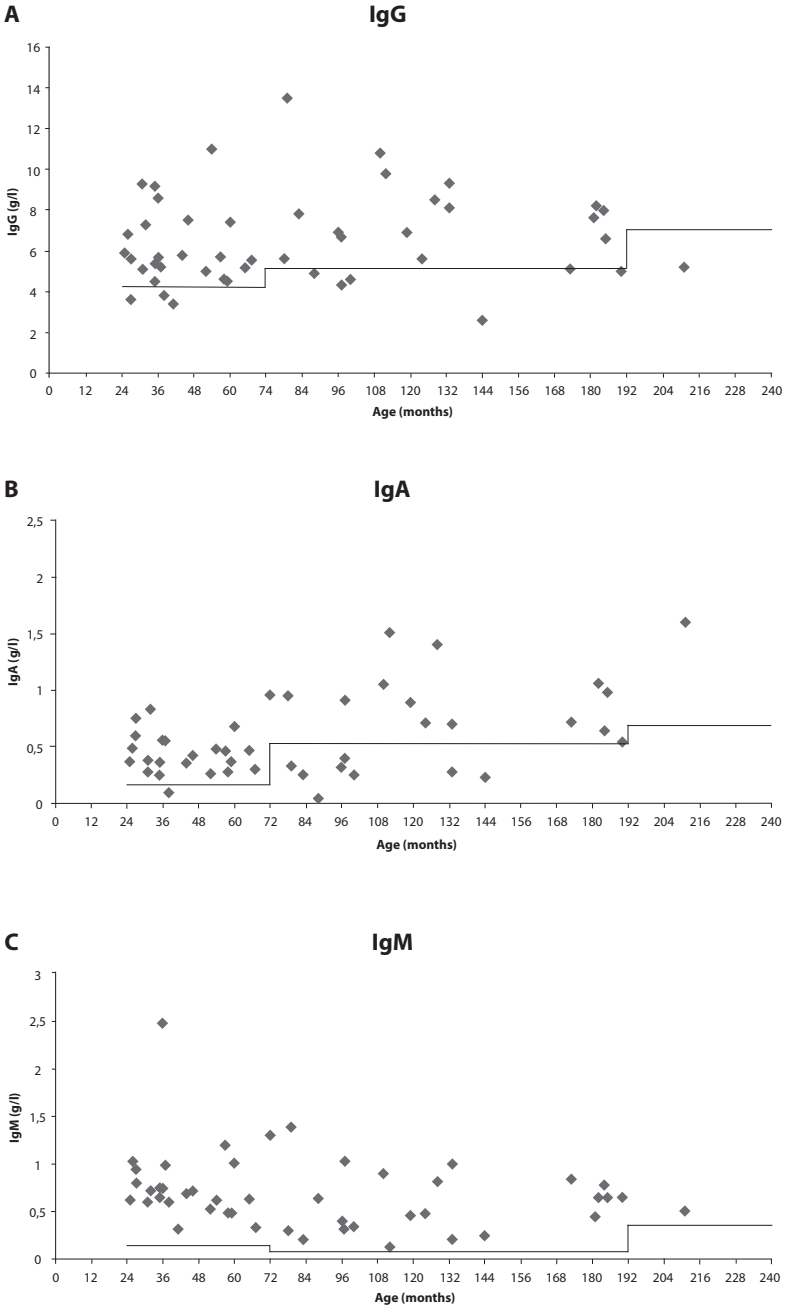

Figure 3
Levels of Immunoglobulin isotypes. **A**: IgG in g/l; **B**: IgA in g/l; **C**: IgM in g/l.
Every dot represents a patient. The bold black line is the lower limit of normal according to age-matched reference values (*ref de Vries E. Clin Exp Immunol 2012;167(1):108-19*).

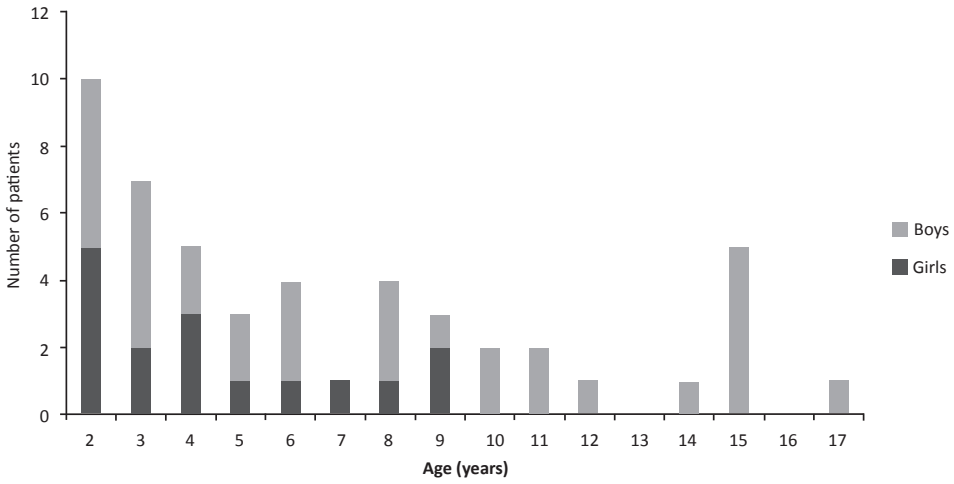

Figure 4.
Distribution of age and gender

Discussion

The human IgG-subclasses were first described in the 1960's by Terry and Fahey [19] and the first report of patients with IgG-subclasses deficiency was published in 1974 [20]. Since then, several cases and cohorts of patients with IgG-subclass deficiency have been described (for example [21]). It was found that co-occurrence of IgA and IgG-subclass deficiency leads to more and more severe recurrent infections [22-24]. Also, children with IgG2-subclass deficiency showed decreased responses to immunization with polysaccharide antibodies [25].

We describe a cohort of 49 children with IgG-subclass deficiency and/or SPAD collected from secondary and tertiary hospitals all over the Netherlands using the Netherlands Pediatric Surveillance Unit system. The majority of the children (69%) in our cohort were reported by four secondary hospitals with a pediatric immunologist in the staff. Our cohort contained only 1 child from a tertiary center. This means that most children with IgG-subclass deficiencies and/or SPAD are not referred to a tertiary center (at least in the Netherlands), probably because their disease is not considered to be very severe. It is unlikely that children with IgG-subclass deficiency and/or SPAD live in clusters around pediatric immunologists practising in secondary hospitals. Apparently, children with IgG-subclass deficiency and/or SPAD are often not recognized by general pediatricians. But is this important? Young children with 'milder' antibody deficiencies often recover. What they show is in fact a delayed maturation of the immune system, transient hypogammaglobulinemia of infancy, not an intrinsic immunodeficiency. This is seen in IgG-subclass deficiency and/or SPAD, but also in IgA deficiency, where 23% of the patients diagnosed with IgA deficiency at the age of 4 years achieve serum IgA levels

above 0.07 g/L during follow-up [26]. However, in some children the immunodeficiency persists or even develops into a more severe form, and recurrent pulmonary infections can lead to chronic pulmonary damage [27]. A study among 55 patients (27 children and 28 adults) with specific antibody deficiency or CVID showed that immunoglobulin class concentrations do not predict bronchiectasis, but percentages and absolute numbers of $CD19^{+}CD27^{+}IgD^{-}$ memory switched B cells do [28]. This was not determined in most patients in our cohort, but 5 children (10%) in our cohort showed bronchiectasis at the time of inclusion. In three of them follow-up data were available; none had improved, and one had developed full-blown CVID. Also, significantly more boys (67%) than girls were reported. After the age of 9, only boys were reported, and only boys (11/33 boys at baseline) showed progressive immunodeficiency during follow-up. This confirms the predominance of boys as was described by us before in a European cohort of children with hypogammaglobulinemia from the ESID online Registry (63% male). Boys with hypogammaglobulinemia in the ESID online Registry were younger at diagnosis than girls and suffered more disease complications [2]. This is in contrast to the paper by Boyle et al. who did not find male sex to be a risk factor for developing SPAD [9]. Furthermore, van Winkle et al. showed that transient hypogammaglobulinemia resolved more quickly in male infants [29], but that cohort did not include older children. This gender difference is an important issue. Unidentified X-linked disease may play a role in boys with antibody deficiency; if so, this should influence treatment decisions, and it has consequences for potential recurrence in the family.

Twenty-five percent of the children with IgG-subclass deficiency and/or SPAD in our prospectively followed cohort recovered during follow-up. This is less than seen in other, retrospective studies of children with IgG-subclass deficiency (recovery 30-67%) or unclassified hypogammaglobulinemia (recovery 49%) [11, 30, 31]. However, follow-up data for our cohort were available in only half the children. This may have lead to an underrepresentation of recovered children during follow-up, because parents of children without persisting medical problems are probably less motivated to participate in a follow-up study.

The most prevalent IgG-subclass deficiency in our cohort was IgG2 deficiency: 24 children had isolated IgG2 deficiency, 5 children had IgG1 and IgG2 deficiency, 5 children had IgG2 and IgG3 deficiency and 3 children had IgG1, IgG2 and IgG3 deficiency. This is in contrast with other published cohorts: IgG3 deficiency was most prevalent in cohorts of Thai and Turkish children with IgG-subclass deficiency [11, 12, 30]. In the Turkish studies 78-90%, and in the Thai study 86% of the patients received prophylactic treatment to prevent infections, versus 47% in our cohort, suggesting a more severe phenotype in these tertiary populations. Additional data in a broad, much larger cohort are needed to assess whether IgG3 deficiency is indeed more severe than IgG2 deficiency; the ESID online Registry would be a good tool for this.

Anti-polysaccharide antibodies were only examined in 21 children in our cohort (14 children from a secondary hospital with a pediatric immunologist in the staff). This means that 28 children did not undergo a complete analysis. The clinical relevance of selected

IgG-subclass deficiency is controversial and determining the ability to produce specific antibodies is considered to be more important [32]. IgG-subclasses can however easily be determined in daily practice with a single venipuncture during the patient's visit to the clinic. The production of specific antibodies requires two venipunctures and vaccination. Also, the interpretation of the vaccine response is difficult; it requires sufficient experience, since reference values are lacking.

Our study has several limitations. We collected our cohort by using the Netherlands Pediatric Surveillance Unit system. Therefore, we depended on the pediatricians in the different hospitals for the quality of the reported data. We could not confirm the diagnosis of IgG-subclass deficiency and/or SPAD in 41 out of 90 (46%) reported children. This was due to missing data in 35 children. In 6 children, all laboratory values were available but normal when using age-matched reference values. Possibly, their pediatricians had used different, non-age-specific, reference values. This is an important issue for educational improvement.

Finally, IgG subclass deficiency can also be found in asymptomatic patients. Analysis of IgG subclasses among 8015 adult blood donors demonstrated that there were more individuals with low IgG2 concentrations than predicted by the log-normal distribution [33]. Also, 11 out of a cohort of 575 healthy children showed IgG2 concentrations >2SD below the mean for age. These asymptomatic children with subnormal IgG2 levels showed normal antibody responses to immunization with Haemophilus influenza type B polysaccharide antigens [34]. However, the children in our cohort were analyzed by their pediatrician because of their clinical presentation, and therefore were not asymptomatic.

Conclusion

This is the first description of a Western-European cohort of children with IgG-subclass deficiency and/or SPAD collected by pediatricians from secondary as well as tertiary hospitals; it was collected in the Netherlands. We show that IgG-subclass deficiency and/or SPAD are not always benign diseases: 10% of the children already showed bronchiectasis. There was an overrepresentation of boys and only boys showed immunological decline during follow-up. So, unidentified X-linked disease may play a role in boys with antibody deficiency. Most IgG-subclass deficiencies and/or SPAD were reported and managed in secondary hospitals with a pediatric immunologist in the staff, which raises the question if these patients are missed in general pediatric secondary hospitals. If we want to identify more patients, probably the awareness of these diseases among general pediatricians should increase.

Acknowledgements

We would like to thank the following pediatricians for reporting patients: E.J.A. Gerritsen (Admiraal de Ruyter Ziekenhuis), T.C.J. Sas (Albert Schweitzer Ziekenhuis), A.A.P.H. Vaessen-Verberne (Amphia Ziekenhuis), M.L. Brouwer (Canisius Wilhelmina Ziekenhuis), C.J. Miedema (Catharina Ziekenhuis), J. van der Deure (Deventer Ziekenhuis), G.J.A. Driessen (Erasmus MC Sophia), A. Dings-Lammertink (Gelre Ziekenhuizen), I. Heuts-van de Kragt (Ikazia Ziekenhuis), C.R.W. Korver (Kennemergasthuis), R.G.M. Bredius (Leids Universitair Medisch Centrum), A.C. Engelberts (Maaslandziekenhuis), W.P. Vogt (MC Groep), G. Brinkhorst (Medisch Centrum Alkmaar), N. de Vries/T.E. Faber/E. van Pinxteren-Nagler (Medisch Centrum Leeuwarden), E. van den Berg (Medisch Spectrum Twente), A. van Kempen (Onze Lieve Vrouwe Gasthuis), A. Warris (Radboud UMC), G. Meppelink/S. Nowak (Refaja Ziekenhuis), A.W. Colijn/G. Nolles/A.L.T. van Overbeek-van Gils (Scheper Ziekenhuis), H.H. Kiezebrink-Lindenhovius (Spaarne Ziekenhuis), M. van Dongen-van Baal (St. Antonius Ziekenhuis), J.F.M. Bruinenberg (St. Elisabeth Ziekenhuis), J. Raaijmakers (St. Laurentius Ziekenhuis), S. Kapteijns-van Kordelaar (St. Jansgasthuis), A. Kaldi (Talma Sionsbergziekenhuis), B. van Ewijk (Tergooi Ziekenhuizen), J.A. Gerrits-Kuiper (van Weel Bethesda Ziekenhuis), C.A.M. van Wijk (Vlietland Ziekenhuis), S. Broekhuizen-den Haan (Wilhemina Ziekenhuis), pediatricians from Ziekenhuis Amstelland, G.H. van Leeuwen (Ziekenhuis Bethesda), K.A. Zwager-Ankone (Ziekenhuis Coevorden-Hardenberg), A.J. Janse (Ziekenhuis Gelderse Vallei), W. Corijn (Ziekenhuisgroep Zeeuws-Vlaanderen).

References

1. Gathmann B, Grimbacher B, Beaute J, Dudoit Y, Mahlaoui N, Fischer A, et al. The European internet-based patient and research database for primary immunodeficiencies: results 2006-2008. Clin Exp Immunol. 2009;157 Suppl 1:3-11.
2. Schatorje EJ, Gathmann B, van Hout RW, de Vries E. The PedPAD study: boys predominate in the hypogammaglobulinaemia registry of the ESID online database. Clin Exp Immunol. 2014;176(3):387-93.
3. Stiehm RE. The four most common pediatric immunodeficiencies. Adv Exp Med Biol. 2007;601:15-26.
4. Kusters MA, Verstegen RH, Gemen EF, de Vries E. Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol. 2009;156(2):189-93.
5. Sanders LA, Rijkers GT, Tenbergen-Meekes AM, Voorhorst-Ogink MM, Zegers BJ. Immunoglobulin isotype-specific antibody responses to pneumococcal polysaccharide vaccine in patients with recurrent bacterial respiratory tract infections. Pediatr Res. 1995;37(6):812-9.
6. Fleming DW, Cochi SL, Hightower AW, Broome CV. Childhood upper respiratory tract infections: to what degree is incidence affected by day-care attendance? Pediatrics. 1987;79(1):55-60.
7. Glocker E, Ehl S, Grimbacher B. Common variable immunodeficiency in children. Curr Opin Pediatr. 2007;19(6):685-92.
8. Monto AS, Napier JA, Metzner HL. The Tecumseh study of respiratory illness. I. Plan of study and observations on syndromes of acute respiratory disease. Am J Epidemiol. 1971;94(3):269-79.
9. Boyle RJ, Le C, Balloch A, Tang ML. The clinical syndrome of specific antibody deficiency in children. Clin Exp Immunol. 2006;146(3):486-92.
10. Ruuskanen O, Nurkka A, Helminen M, Viljanen MK, Kayhty H, Kainulainen L. Specific antibody deficiency in children with recurrent respiratory infections: a controlled study with follow-up. Clin Exp Immunol. 2012;172(2):238-44.
11. Karaca NE, Karadeniz C, Aksu G, Kutukculer N. Clinical and laboratory evaluation of periodically monitored Turkish children with IgG subclass deficiencies. Asian Pac J Allergy Immunol. 2009;27(1):43-8.
12. Visitsunthorn N, Hengcrawit W, Jirapongsananuruk O, Luangwedchakam V. Immunoglobulin G (IgG) subclass deficiency in Thai children. Asian Pac J Allergy Immunol. 2011;29(4):332-7.
13. Rosen P, Hopkin RJ, Glass DN, Graham TB. Another patient with chromosome 18 deletion syndrome and juvenile rheumatoid arthritis. J Rheumatol. 2004;31(5):998-1000.
14. Elberse KE, Tcherniaeva I, Berbers GA, Schouls LM. Optimization and application of a multiplex bead-based assay to quantify serotype-specific IgG against Streptococcus pneumoniae polysaccharides: response to the booster vaccine after immunization with the pneumococcal 7-valent conjugate vaccine. Clin Vaccine Immunol. 2010;17(4):674-82.
15. Go ES, Ballas ZK. Anti-pneumococcal antibody response in normal subjects: a meta-analysis. J Allergy Clin Immunol. 1996;98(1):205-15.
16. Sanders LA, Rijkers GT, Kuis W, Tenbergen-Meekes AJ, de Graeff-Meeder BR, Hiemstra I, et al. Defective antipneumococcal polysaccharide antibody response in children with recurrent respiratory tract infections. J Allergy Clin Immunol. 1993;91(1 Pt 1):110-9.
17. Janssen WJ, Bloem AC, Vellekoop P, Driessen GJ, Boes M, van Montfrans JM. Measurement of pneumococcal polysaccharide vaccine responses for immunodeficiency diagnostics: combined IgG responses compared to serotype specific IgG responses. J Clin Immunol. 2014;34(1):3-6.
18. Rodenburg GD, Sanders EA, van Gils EJ, Veenhoven RH, Zborowski T, van den Dobbelsteen GP, et al. Salivary immune responses to the 7-valent pneumococcal conjugate vaccine in the first 2 years of life. PLoS One. 2012;7(10):e46916.
19. Terry WD, Fahey JL. Subclasses of Human Gamma-2-Globulin Based on Differences in the Heavy Polypeptide Chains. Science. 1964;146(3642):400-1.
20. Oxelius VA. Chronic infections in a family with hereditary deficiency of IgG2 and IgG4. Clin Exp Immunol. 1974;17(1):19-27.

21. Stanley PJ, Corbo G, Cole PJ. Serum IgG subclasses in chronic and recurrent respiratory infections. Clin Exp Immunol. 1984;58(3):703-8.
22. Oxelius VA, Laurell AB, Lindquist B, Golebiowska H, Axelsson U, Bjorkander J, et al. IgG subclasses in selective IgA deficiency: importance of IgG2-IgA deficiency. N Engl J Med. 1981;304(24):1476-7.
23. Morell A, Muehlheim E, Schaad U, Skvaril F, Rossi E. Susceptibility to infections in children with selective IgA- and IgA-IgG subclass deficiency. Eur J Pediatr. 1986;145(3):199-203.
24. Beard LJ, Ferrante A, Oxelius VA, Maxwell GM. IgG subclass deficiency in children with IgA deficiency presenting with recurrent or severe respiratory infections. Pediatr Res. 1986;20(10):937-42.
25. Umetsu DT, Ambrosino DM, Quinti I, Siber GR, Geha RS. Recurrent sinopulmonary infection and impaired antibody response to bacterial capsular polysaccharide antigen in children with selective IgG-subclass deficiency. N Engl J Med. 1985;313(20):1247-51.
26. Lim CK, Dahle C, Elvin K, Andersson BA, Ronnelid J, Melen E, et al. Reversal of Immunoglobulin A Deficiency in Children. J Clin Immunol. 2014.
27. Ozkan H, Atlihan F, Genel F, Targan S, Gunvar T. IgA and/or IgG subclass deficiency in children with recurrent respiratory infections and its relationship with chronic pulmonary damage. J Investig Allergol Clin Immunol. 2005;15(1):69-74.
28. Alachkar H, Taubenheim N, Haeney MR, Durandy A, Arkwright PD. Memory switched B cell percentage and not serum immunoglobulin concentration is associated with clinical complications in children and adults with specific antibody deficiency and common variable immunodeficiency. Clin Immunol. 2006;120(3):310-8.
29. Van Winkle RC, Hauck WW, McGeady SJ. Phenotypic parameters predict time to normalization in infants with hypogammaglobulinemia. J Clin Immunol.33(8):1336-40.
30. Kutukculer N, Karaca NE, Demircioglu O, Aksu G. Increases in serum immunoglobulins to age-related normal levels in children with IgA and/or IgG subclass deficiency. Pediatr Allergy Immunol. 2007;18(2):167-73.
31. Kutukculer N, Gulez N. The outcome of patients with unclassified hypogammaglobulinemia in early childhood. Pediatr Allergy Immunol. 2009;20(7):693-8.
32. Buckley RH. Immunoglobulin G subclass deficiency: fact or fancy? Curr Allergy Asthma Rep. 2002;2(5):356-60.
33. Madassery JV, Kwon OH, Lee SY, Nahm MH. IgG2 subclass deficiency: IgG subclass assays and IgG2 concentrations among 8015 blood donors. Clin Chem. 1988;34(7):1407-13.
34. Shackelford PG, Granoff DM, Madassery JV, Scott MG, Nahm MH. Clinical and immunologic characteristics of healthy children with subnormal serum concentrations of IgG2. Pediatr Res. 1990;27(1):16-21.

CHAPTER 6

Primary Immunodeficiency associated with Chromosomal Aberration - An ESID Survey

Orphanet J Rare Dis. 2016 Aug 2;11(1):110.

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Abstract

Background

Patients with syndromic features frequently suffer from recurrent respiratory infections, but little is known about the spectrum of immunological abnormalities associated with their underlying chromosomal aberrations outside the well-known examples of Down and DiGeorge syndromes. Therefore, we performed this retrospective, observational survey study.

Methods

All members of the European Society for Immunodeficiencies (ESID) were invited to participate by reporting their patients with chromosomal aberration (excluding Down and DiGeorge syndromes) in combination with one or more identified immunological abnormalities potentially relating to primary immunodeficiency. An online questionnaire was used to collect the patient data.

Results

Forty-six patients were included from 16 centers (24 males, 22 females; median age 10.4 years [range 1.0-69.2 years]; 36 pediatric, 10 adult patients). A variety of chromosomal aberrations associated with immunological abnormalities potentially relating to primary immune deficiency was reported. The most important clinical presentation prompting the immunological evaluation was 'recurrent ear-nose-throat (ENT) and airway infections'. Immunoglobulin isotype and/or IgG-subclass deficiencies were the most prevalent immunological abnormalities reported.

Conclusions

Our survey yielded a wide variety of chromosomal aberrations associated with immunological abnormalities potentially relating to primary immunodeficiency. Although respiratory tract infections can often also be ascribed to other causes (e.g. aspiration or structural abnormalities), we show that a significant proportion of patients also have an antibody deficiency requiring specific treatment (e.g. immunoglobulin replacement, antibiotic prophylaxis). Therefore, it is important to perform immunological investigations in patients with chromosomal aberrations and recurrent ENT or airway infections, to identify potential immunodeficiency that can be specifically treated.

Background

'Syndromic' patients frequently suffer from recurrent respiratory infections; it is a major cause of morbidity and mortality in this patient group. However, in these patients immunological work-up is often not performed because an immunodeficiency is not suspected. The infections are often ascribed to food and saliva aspiration [1], structural abnormalities of the upper respiratory tract, neuromuscular problems, malnutrition or institutionalization. Besides, other problems are often more prominent than the recurrent infections. This may lead to underdiagnosis of 'syndromic immunodeficiency'. However, identification of an underlying immune defect may be therapeutically actionable, which in turn may improve the quality of life in these patients: for instance hypogammaglobulinemia can be treated with immunoglobulin replacement [2,3]. In addition, information regarding genes critical for the development and functioning of the immune system may be gained by analyzing the precise chromosomal defect and the concomitant immunological phenotype.

Several primary immunodeficiency (PID) disorders have been identified and increasingly their genetic backgrounds have been unraveled [4]. Syndromes with chromosomal abnormalities of number or structure are considered as a distinct group within PID [5]. Clear examples are Down syndrome (trisomy 21) [6] and DiGeorge syndrome (22q11 deletion) [7]. Also, Turner syndrome [8] and Wolf-Hirschhorn syndrome [9] are known to be associated with immunodeficiency. In the past ten years, thirteen cases, three patient series and two families with other chromosomal aberrations and immunological abnormalities have been described in the literature [10-27]. There is one study that screened patients with dysmorphic disorders for immune defects. They showed a high incidence of immunodeficiency in this population (23 out of 29 patients had one or more defects); however, they also included 11 patients with Down syndrome [28]. We hypothesize that in patients with chromosomal aberrations, other than the well-known Down and DiGeorge syndromes, concomitant 'syndromic' immunodeficiency may be underdiagnosed. To unequivocally prove this, a large case-control study would be needed; this is not really feasible. To explore this further, we performed a retrospective, observational survey study.

Methods

An email message with the proposal to participate in a survey study was sent out to all members of the European Society for Immunodeficiencies (ESID) to identify as many patients known to ESID members as possible with a chromosomal aberration in combination with one or more identified immunological abnormalities relating to PID. Exclusion criteria were trisomy 21 (Down syndrome) and 22q11 deletion (DiGeorge syndrome), because the immunological abnormalities in these syndromes have been described in detail before [6, 7]. Those ESID members who agreed to participate in the

study were requested to complete an online questionnaire for each of their eligible patients (Additional File 1). The patients were identified by physician recall. The answers to the questionnaires were encrypted and saved on a protected server; these data did not contain any information that enabled identification of the identity of the patients. Clinical characteristics and identified immunological abnormalities were reported. Age-matched reference values were used for interpretation of immunoglobulin levels and lymphocyte subpopulation counts; values below the age-matched reference values were scored as 'low' [29-31]. For the interpretation of the vaccine responses (i.e. before and after diagnostic vaccination with Tetanus and PneumoVax® or Pneumo(vax)23®) reference values from the laboratory performing the tests were used. For responses to Pneumovax® or Pneumo(vax)23® measured by serotype, a titer ≥1 IU/ml per serotype was considered to be a sufficient response. If only total IgG for S. pneumoniae was tested, a >4 fold increase of titer was considered as a positive response. Additional immunological tests were performed judged necessary by the treating physician and are therefore only available for some patients. Lymphocyte function tests included in vitro T-lymphocyte proliferation tests (to Concanavalin A (ConA), phytohaemagglutinin (PHA), pokeweed mitogen (PWD) and Staphylococcus aureus enterotoxin A (SAE)), natural killer (NK) cell and cytotoxic T cell toxicity (in vitro stimulated CD107a degranulation). Granulocyte function tests included oxidative burst, the quantitative nitroblue tetrazolium dye reduction (NBT) test and phagocytosis test (cells Escherichia coli opsonised). For these additional immunological test (e.g. lymphocyte and granulocyte function tests), the laboratory-specific reference values were used. Furthermore, we asked all the participating centers to provide us with the number of patients with chromosomal aberrations who had undergone an immunological evaluation but were subsequently found *not* to have an immunological abnormality. This was also based on physician recall. Descriptive statistics were performed. The International System for Human Cytogenetic Nomenclature 2013 (ISCN) was used for cytogenetic nomenclature [32]; an overview is given as a group, and in relation to the specific chromosomal aberrations concerned. The Medical Ethical Committee Brabant approved of the study procedures.

Results

Fifty-two patients from 16 different centers distributed globally were reported. Six patients had to be excluded because they did not meet the inclusion criteria: 3 patients did not have a confirmed chromosomal aberration, 1 patient with Rubinstein-Taybi Syndrome (no chromosomal aberration, only single gene mutation), 1 patient with suspected Kabuki Syndrome (no genetic diagnosis) and 1 patient with Rothmund-Thomson Syndrome (no chromosomal aberration, only single gene mutation). Three other patients did not have an immunodeficiency, these were 3 related patients with familial t(12;14). An overview of the excluded patients is presented in Additional File 2.

The 46 included patients consisted of 24 males and 22 females with a median age of 10.4 years at the moment of reporting (range 1.0-69.2 years; 36 pediatric and 10 adult patients). Two families were reported: patients 17 and 20 are related, as well as patients 18 and 19 (they are also related to the excluded patients 2, 3 and 4, see Additional File 2). Fifteen of these 46 included patients have been published before and publication of two patients is currently in press (for details see Table 1).

Seven out of the total 16 centers provided the number of patients with chromosomal aberrations who had undergone an immunological evaluation but were subsequently found *not* to have an immunological abnormality. Together, they reported 27 patients with immunological abnormalities in this survey; they also reported 63 patients with chromosomal aberrations in whom immunological assessment revealed no abnormality. Thus, of these centers 30% of the patients with chromosomal aberrations who underwent immunological evaluation were diagnosed with some form of primary immunodeficiency.

Symptoms indicative of PID can be divided into eight different clinical presentations [29]; 'recurrent ear-nose-throat (ENT) and airway infections' were most commonly reported in this cohort (in 43/46 patients). In 31/46 patients, 'recurrent ENT and airway infections' was reported as the clinically most important presentation. Other PID-related manifestations reported as the most important clinical presentation include 'auto-immune or chronic inflammatory disease; lymphoproliferation'(n=5); 'failure to thrive from early infancy' (n=4); 'unusual infections or unusually severe course of infections'(n=3); 'recurrent pyogenic infections' (n=2) and 'recurrent infections with the same type of pathogen'(n=1). The most common syndromic-related manifestations were: developmental delay (n=37), ataxia, paresis or other motor disability (n=16), dysmorphic features (n=31), microcephaly (n=11), growth retardation (n=19), atopic eczema (n=8), hair and/or nail abnormalities (n=1) and hypopigmentation (n=1).

A detailed overview of the clinical findings is shown in Table 1.

Antibody deficiency was the most common immunological defect identified. Of 33 patients reported to have low immunoglobulin isotype(s), 20 had low IgG (Figure 1). Nine patients had low IgG with completely absent IgA and 8 patients had low IgG in combination with low IgM. IgG subclass deficiency was identified in 18 patients, of which 15 had concomitant low total immunoglobulin isotype(s) and 3 did not. Vaccine responses were tested in 32/46 patients and were found insufficient in 18 patients: 16/18 were insufficient for pneumococcal polysaccharide vaccine. Four patients (no. 2, 22, 23 and 24) showed normal antibody production after diagnostic vaccination despite low serum immunoglobulins. For patients 23 and 24, however, the decreased response was based on total IgG for S. pneumoniae. Responses to live vaccines were not documented; no unfavorable outcomes of natural chickenpox infection were reported. 18/46 patients were treated with immunoglobulin replacement. The indication of immunoglobulin replacement therapy was based on clinical grounds, as judged by the treating physician. In 2 patients lymphopenia was reported; one of them was neutropenic as well. Lymphocyte subpopulations (CD3, CD4, CD8, CD19 and CD16/56) were determined in 36 patients; in 16 a decreased count of $\geq$1 (sub)populations was reported. In 11 patients more extensive B cell subpopulations were determined and

in 7 patients extensive T cell subpopulations (protocols differed per patient). In 1 patient total absence of B lymphocytes was reported (patient number 5; trisomy 13). Lymphocyte and granulocyte function tests were performed in 11 and 11 patients, respectively; in 2 decreased lymphocyte as well as granulocyte function was reported (patients 2 and 3). A detailed overview of the immunological and other laboratory findings is presented in Table 2 and Additional File 3.

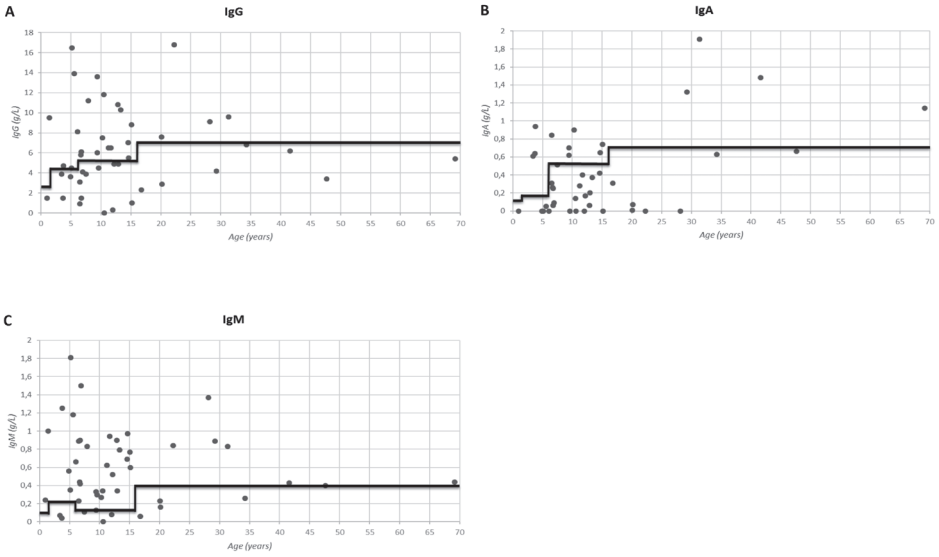

Figure 1. Levels of immunoglobulin isotypes. Every dot represents a patient. The bold black line is the lower limit of normal according to age-matched reference values (ref de Vries E. Clin Exp Immunol 2012;167(1):108-19.) **A.** IgG in g/L. **B.** IgA in g/L; two values > 2.0 g/l are not displayed in the graph. **C.** IgM in g/L

Discussion

Our call identified 46 patients with chromosomal aberration associated with immunodeficiency, the largest cohort reported in the literature so far (42 isolated cases, and twice 2 patients from the same family). Based on data from 7/16 participating centers, up to one third of patients with chromosomal aberrations and recurrent infections may have some form of primary immunodeficiency. Because the patients in this study were identified by physician recall, reporter bias is possible. However, the relative number is much higher than the 6% found in a cohort of 259 'normal' children screened for immunological abnormalities because of recurrent infections by Brodszki et al. [33]. The most common clinical presentation in our cohort was 'recurrent ENT and airway infections', which triggered their physician to perform immunological investigations.

Table 1. Clinical characteristics of the included patients.

Nr	Sex	Age (yrs)[1]	Genetics	Immunological presentation[2]	Other clinical presentations[3]	Other symptoms
1	M	15.2	46,XY,dup(6)(p12.2p21.31)	Airways	Developmental delay Dysmorphic features Microcephaly	Prematurity 36 weeks Tracheostomy Feeding difficulties Infantile pyloric stenosis Pulmonary congestion Intractable diarrhoea
2[a]	M	3.4	46,XY.ish der(16)t(16;19)(p13.3;p13.3) arr[hg19] 16p13.3(106 271-1 024 153)x1, 19p13.3(327 273-6 887 622)x3	Failure to thrive	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Microcephaly Growth retardation	Bilateral inguinal hernia Horse shoe kidney Hypospadia, hydrocele Maldescensus testis
3[a]	M	9.4	46,XY.ish der(14)t(14;19)(p11.2;p13.2) de novo; arr[hg19] 19p13.3p13.2(90 897-7 300 043)x3	Unusual infections	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Microcephaly Growth retardation	Bilateral incarcerated inguinal hernia Congenital hip dysplasia Perineal hypospadia/penoscrotal fistula Severe osteopenia Sensorineurinal hearing loss Epilepsy
4	M	5.6	46,XY,del(18)(p11.2)	Airways	Ataxia, paresis or other motor disability Growth retardation	na
5	M	12.0	47,XY,+13	Airways	Developmental delay Growth retardation	Sepsis Seizures Gastroesophageal reflux disease Loss of vision
6	F	6.1	46,XX,del(16)(p11.2)	Airways	Developmental delay Dysmorphic features	Obesity Autism BCGosis
7	M	5.1	46,XY,del(2)(q33.2)	AI disease	Developmental delay Dysmorphic features	Cleft palate PDA Splenomegaly Auto-immune hemolytic anemia
8	M	1.0	*No full karyotype available* Array CGH : gain of 144kB in 9p24.3 and loss of 15MB in 10q26.11.q26.3	Unusual infections	Developmental delay Dysmorphic features Microcephaly Growth retardation	Duodenal atresia PDA Micropenis, gonadal agenesia
9	F	5.2	46,XX,del(18)(q22)	AI disease	Developmental delay Dysmorphic features	Auto-immune polyendocrine syndrome type II with: Thyroiditis Vitiligo Pernicious anemia Type 1 diabetes mellitus
10	F	1.4	46,XX,arr[hg19] 16p11.2 (29,567,295-30,177,916)x1 dn	Failure to thrive	Developmental delay Growth retardation	Recurrent fever
11	F	69.2	45,X	Airways	Dysmorphic features Growth retardation	Schwannoma Hearing loss
12	F	6.5	45,X[42]/47,XXX[8]	Airways	Developmental delay Growth retardation	Currarino syndrome
13	F	41.6	45,X	Unusual infections	Growth retardation	na
14	M	20.1	46,XY,der(X)t(X;18)(q28;q23) (MECP2 duplication)	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features	Vitiligo Bronchiectasis Small intestinal villous atrophy
15[b]	M	7.5	46,XY,r(18)(p11.2q23)[97]/45,XY,-18[3]	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Microcephaly Growth retardation	ASD II, VSD Micropenis
16[c]	F	47.7	arr[hg19] 11q24.2q25 (126,074,297-134,927,114)x1	Airways	Developmental delay Dysmorphic features Atopic eczema	VSD Infertility HPV associated giant condylomata Hypothyroid Idiopathic angio-edema Severe asthma Hypersplenism Obesity, type II diabetes Bronchiectasis
17[d]	M	22.3	46,XY,der(18)t(10p;18q) with 18q22.3–q23 deletion and partial trisomy of 10pter	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Growth retardation	Hypothyroid (subclinical) Pulmonary valve stenosis

Table 1. continued

Nr	Sex	Age (yrs)[1]	Genetics	Immunological presentation[2]	Other clinical presentations[3]	Other symptoms
18[e]	F	29.3	46,XX,t(12;14)(p11.2;q13)	AI disease	Atopic eczema	Samter's triad* ALL Migraine Recurrent herpes labialis HPV associated condylomata Multiple allergies
19[e]	F	4.9	46,XX,t(12;14)(p11.2;q13)	Airways	None	na
20[d]	F	28.2	46,XX,der(18)t(10p;18q) with 18q22.3–q23 deletion and partial trisomy of 10pter	Airways	Ataxia, paresis or other motor disability Dysmorphic features Atopic eczema	Thymus hyperplasia Atopy Polyarticular JIA
21	F	31.3	46,XX,arr[hg19] 15q25.2 (83,214,012-84,776,990)x1	Airways	None	Allergy Epilepsy Asthma Cholesteatoma Recurrent monoarthritis
22	M	34.3	46,XY,inv(10)(q21q23)	AI disease	None	Asymptomatic
23[f]	F	6.9	46,XX,del(19)(p13.13)	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Microcephaly Growth retardation	IUGR Epilepsy
24[f]	M	9.6	46,XY,r(18)	AI disease	Developmental delay Dysmorphic features Growth retardation Hypopigmentation	Panniculitis with lipodystrophy Auto-immune hypothyroidism Vitiligo Chronic urticaria Subaortic stenosis
25[g]	M	16.8	46,XY,der(11)dup(11)(q22q23) del(q24.3)	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Atopic eczema Hair and/or nail abnormalities	na
26	M	3.7	*No full karyotype available* arr[hg19]11p12-p11.12 (38.090.281-49.257.082)x1	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features	Defective absorption folinic acid
27	F	10.3	46,XX,del(11)(q11)	Failure to thrive	Developmental delay Dysmorphic features Growth retardation Atopic eczema	na
28	F	5.9	49,XXXXX	Pyogenic infections	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features	PS and ASD Hypermobility Radio-ulnar synosthosis
29	M	15.1	46,XY.ish del(X)(p11.3p11.3) (RP4-628F15+,RP11-245M24 dim,RP6-99M1-,RP4-689N3-,RP11-1409+)mat	Failure to thrive	Developmental delay Microcephaly Growth retardation	Visual impairment Retinitis pigmentosa
30	M	9.4	46,XY,r(6)	Airways	Developmental delay Dysmorphic features Microcephaly Growth retardation	Gastro-oesofageal reflux Dilated cardiomyopathy and small VSD
31[h]	F	12.8	46,XX,del(18)(p11.1)	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Growth retardation	Type I diabetes mellitus Growth hormone deficiency Autoimmune thyroiditis Pectus excavatum Retrognathia with absent maxillary chondyles
32	M	6.8	46,XY,del(7)(q22.3 q31.3)	Airways	Developmental delay Dysmorphic features	na
33	F	14.7	47,XX,+der(22)t(11;22) (q23;q11) mat (partial trisomy 11q)	Airways	Developmental delay Ataxia, paresis or other motor disability	Palatoschizis, preauricular tags Anus atresia Urolithiasis
34	F	7.9	46,XX.arr snp 2p23.1(SNP_A-2078092->SNP_A-2248377)x1 mat	Same pathogen	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features	Mitochondrial dysfunction Acracyanosis Bronchiectasis Hyposplenia
35	M	11.3	46,XY.arr[hg19] 3p14.3(57,994,310-58,071,249)x1 pat	Airways	Developmental delay Dysmorphic features	Submucosal palatal schisis Transient neonatal macroglossi Hepatosplenomegaly

Table 1. continued

Nr	Sex	Age (yrs)[1]	Genetics	Immunological presentation[2]	Other clinical presentations[3]	Other symptoms
36	F	6.8	45,X	Airways	None	na
37	F	20.2	46,XX,der(2)t(2;10)(q37.3;q26.3)mat.arr snp 2q37.2q37.3(SNP_A-1957498->SNP_A-2027809)x1,10q26.3(SNP_A-2264115->SNP_A-1934598)x3	Airways	Developmental delay Ataxia, paresis or other motor disability	Autistiform developmental delay Splenomegaly Cytopenias Granulomata Gastroparesis Obesitas
38[i]	M	6.5	49,XXXXY	Airways	Developmental delay Dysmorphic features	na
39[i]	M	10.6	49,XXXXY	Airways	Developmental delay Dysmorphic features	na
40[i]	M	14.6	49,XXXXY	Airways	None	na
41[i]	M	13.3	49,XXXXY	Airways	Developmental delay Dysmorphic features	na
42[i]	M	11.7	49,XXXXY	Airways	Developmental delay Dysmorphic features Atopic eczema	na
43	M	12.2	47,XYY,dup(22)(q11.21)	Pyogenic infections	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Microcephaly	Asthma
44	F	3.8	46,XX.arr snp 1q44(SNP_A-2136114->SNP_A-4223408)x1 dn, 11p11.2p11.12 (SNP_A-1817808->SNP_A-4198132)x3 pat	Airways	Developmental delay Dysmorphic features Microcephaly Growth retardation Atopic eczema	Epilepsy Rocker bottom foot Cow's milk allergy Feeding difficulties
45	F	6.7	46,X,idic(X)(p11.21).arr snp 22q11.21(SNP_A-2108791->SNP_A-2160861)x3 mat, Xp22.33p11.21(SNP_A-4207883->2247707)x1 dn, Xp11.21q28(SNP_A-4201150->SNP_A-2267820)x3 dn	Airways	Developmental delay Growth retardation	Prematurity; gestational age 30 weeks Bone anchored hearing aid Periorbital hemangioma
46	F	12.9	46,XX,arr cgh 16p13.11p13.12 (14,687,636-16,452,200) x3.	Airways	Developmental delay Ataxia, paresis or other motor disability Dysmorphic features Microcephaly Growth retardation Atopic eczema	Severe scoliosis Seizures Myopathy of unknown etiology Chronic progressive external ophthalmoplegia Contractures; wheelchair bound

Headings
Nr= patient number; [1]at the time of reporting; [2]most prominent clinical immunological presentation; [3]other clinical presentations as requested in the survey (Additional File 1).
Patients
(a) previously published in Seidel MG, Duerr C, Woutsas S, et al. J Med Genet 2014;51:254-263, (b) previously published in Celmeli F, et al. J Investig Allergol Clin Immunol. 2014;24(6):442-4, (c) previously published in Seppänen et al. J Clin Immunol 2014;34:114–118., (d) family members and previously published in Dostal et al. International Journal of Immu-genetics 2007;34: 143–147 : patient 17 as IV:4 and patient 20 as IV, (e) family members, together with excluded patient 2, 3 and 4, (f) publication in press, Calvo Campoverde K, et al. Allergologia et Immunopathologia 2016, (g) previously published in Fernandez-San Jose C, J Paediatr Child Health 2011;47(7):485-6, (h) previously published in Browning MJ, J Investig Allergol Clin Immu-l 2010;20(3):263-266, (i) previously published in Keller MD, et al. Am J Med Genet C Semin Med Genet. 2013;163C(1):50-4.
Clinical presentations
Airways = Recurrent ENT and airway infections; FTT = failure to thrive from early infancy; unusual infections = unusual infections or unusually severe course of infections; AI disease = autoimmune or chronic inflammatory disease, lymphoproliferation; pyogenic infections = recurrent pyogenic infections; same pathogen = recurrent infections with the same type of pathogen (de Vries E. Clin Exp Immunol 2012;167(1):108-19).
Other abbreviations
ALL: acute lymphatic leukemia, ASD: atrial septum defect, BCG: Bacillus Calmette-Guérin, F: female, HPV: human papilloma virus, IUGR: intra uterine growth retardation, JIA: juvenile idiopathic arthritis, M: male, na: not available, PDA: patent ductus arteriosus, PEG: percutaneous endoscopic gastrostomy, PS: pulmonary stenosis, VSD: ventricular septum defect, yrs: years.
* Samter's triad: asthma, aspirin and NSAID sensitivity, and nasal/ethmoidal polyposis

Not surprisingly, these were mostly 'predominantly antibody deficiencies'[34] ranging from IgG-subclass deficiency and/or polysaccharide antibody deficiency to severe hypogammaglobulinemia or even agammaglobulinemia in one patient. While this study may have limitations inherent to a retrospective, observational survey (e.g. recall bias; reporting bias; convenience sampling), these findings suggest that 'syndromic immunodeficiency' may be under-diagnosed.

A previous study in patients with dysmorphic features found low $CD19^+$ and $CD16^+$ and/or $CD56^+$ cells as the most frequent immunological abnormalities, followed by low immunoglobulins [28]. However, in contrast to our survey this study also included a lot of patients with Down syndrome (11/29 patients) who are known to have lower $CD19^+$ and $CD3^-CD16^+$ and/or $CD56^+$ cells [6], precluding an appreciation for the possibility of underlying immunodeficiency in patients with non-Down, chromosomal syndromes. Until now, no other cohorts of patients with different chromosomal aberrations associated with immunological abnormalities have been described. The chromosomal aberrations described in our study may provide insight regarding novel genes involved in the immune system, either located directly within or adjacent to the anomalous loci. Several of the cytogenetic abnormalities in our patients have been linked to immunodeficiency or -dysregulation in the literature before.

The largest family in our cohort consists of 5 affected patients with 46,XX,t(12;14)(p11.2;q13) (patients 18, 19 and excluded patients 2, 3 and 4). Only two had immunodeficiency (patients 18 and 19), both with low IgG-levels and one with additional IgA-deficiency and decreased numbers of $CD19^+$ and $CD3^-CD16^+$and/or$CD56^+$ cells. All patients in this family suffered from atopy, asthma and/or allergy (some with anaphylaxis); two developed acute lymphatic leukemia. A candidate gene located on chromosome 14q13 is *nuclear factor of kappa light chain gene enhancer in B cells inhibitor alpha (NFKBIA)* (OMIM 164008). NFKBIA inactivates NF-kappa-B by trapping it in the cytoplasm. Functional impairment of NFKBIA can result in increased activation of the NF-kappa-B pathway leading to immune dysregulation [35].

The other family in our cohort consists of two cousins with an unbalanced translocation t(18q;10p), namely t(18q-;10p+) (patients 17 and 20), effectively resulting in a 18q22.3–q23 deletion and a partial trisomy of 10pter. Both showed IgA-deficiency and IgG-subclass deficiency (both IgG4 and one also IgG2), and one showed decreased numbers of $CD3^-CD16^+$and/or$CD56^+$ cells. One of the cousins showed diffuse thymic hyperplasia (patient 20) without evidence of developing thymoma. Although patients with complete 10p trisomy are not reported to have immunodeficiency [36, 37], patients with terminal deletions of 10p have been reported with IgA- and IgG-deficiency before [21, 38]. The 18q– syndrome is associated with IgA-deficiency and other autoimmune or immunodeficiency diseases, such as common variable immunodeficiency (CVID) [39], juvenile rheumatic arthritis [40], insulin-dependent diabetes mellitus [41], celiac disease [42] and thyroid hormone abnormalities [43]. This partly matches the clinical phenotypes of our related patients. The other patients in our cohort with chromosome 18q aberrations all but one also showed IgA-deficiency (patients 9, 14, and 20). The two cousins from

our study are part of a Finnish family with t(18q;10p), which was published in 2007 [20]. All members of this family showed IgA-deficiency; IgG-subclasses were not tested in the other family members. The authors hypothesized that the observed IgA-deficiency may result from haplo-insufficiency of one or multiple genes located in the 18q22.3–q23 region in possible connection with a larger polygenic network.

Our cohort contains two patients with ring chromosome 18 (one mosaic (patient 15) and one with complete chromosome 18 deletion (patient 24)) and one patient with 18p deletion (patient 31). Deletions of chromosome 18p have also been associated with immune-related dysfunction like autoimmune thyroiditis, diabetes mellitus, IgA deficiency, atopic skin conditions, juvenile rheumatoid arthritis [12, 15, 22, 25], and in one patient with SLE [12]. This matches with our patients: two patients had an IgA-deficiency and the patient with 18p deletion had multiple endocrine dysfunctions. However, our patient with a mosaic form of ring chromosome 18 (46,XY,r(18)(p11.2q23)[97]/45,XY,-18[3], patient 15) showed only low IgM with recurrent respiratory tract infections, as published before [27].

Four of our patients showed chromosome 11q deletions (patients 16, 25, 27 and 33); two of them were published before [14, 26]. Terminal deletion of chromosome 11 can cause Jacobsen syndrome [44] and has previously been associated with hypogammaglobulinemia, pancytopenia and low T-helper cell counts [45, 46]. Our patients with 11q deletion did not show neutropenia or lymphopenia, but three of them had both IgG- and IgA-deficiency. No low T-helper cell counts were reported.

Two centers reported a patient with deletion of chromosome 16p11.2 (patients 6 and 10). Deletions in this region of chromosome 16 are associated with intellectual disability, congenital anomalies, obesity, macrocephaly, and autism [47]. This matches the clinical picture of one of our patients. Both patients showed only minor immunological abnormalities: IgA deficiency and global lymphocytosis. Recently, single nucleotide polymorphisms at the fused-in-sarcoma (FUS)/integrin CD11b (ITGAM) locus at 16p11.2 were associated with CVID phenotypes [48]. In the literature, there is also a report of an autistic girl with a 16p11.2 deletion who also had severe combined immunodeficiency (SCID) caused by Coronin-1A deficiency (also located at 16p11.2) [49]. Coronin-1A is essential for development of a normal peripheral T cell compartment in mice as well as men [33, 50]. However, this girl had, in contrast to our patient, next to the 16p11.2 deletion, also a 2 bp deletion of the Coronin-1A gene on the other (paternal) allele.

Several patients with X-chromosome aberration were included. Turner syndrome (45,X) is known to be associated with immunodeficiency [5, 8], but with different clinical presentations. Our four Turner patients (patients 11, 13 and 36, and patient 12 with mosaicism Turner) also showed a variety of immunological abnormalities. The relationship, if any, between the immune defects in Turner syndrome and those in established X-linked PID remains unknown. Additionally, 5 boys with 49,XXXXY (patients 38, 39, 40, 41 and 42) and 1 girl with 49,XXXXX (patient 28) were reported. The 49,XXXXX girl presented with pyogenic infections and low IgG and IgM levels, but with normal granulocyte levels and function. The 49,XXXXY boys all presented with 'recurrent ENT and airway infections', and they all showed impaired antibody responses to pneumococcal polysaccharide antigens, as was published before [11].

Table 2. Results of immunological tests in the included patients.

Nr	NP	LP	↓G	↓A	↓M	↓IgG subclass		Lymphocyte subsets
1	-	-	+	+*	-	na		↑ aCD3, ↑ aCD3CD4
2[a]	-	-	+	-	+	+	IgG1, IgG3	↓ aCD3, ↓ a smB
3[a]	-	-	-	-	-	-		Borderline ↓ a smB
4	-	-	-	+	-	na		na
5	-	-	+	+*	+	+	IgG1, IgG2, IgG3	Absence of CD19 cells
6	-	-	-	+*	-	-		↑ aCD3, ↑ aCD3CD4, ↑ aCD19
7	-	-	-	+*	-	na		↓ aCD3, ↓ aCD3CD8 ↓ aCD19
8	-	-	+	+*	-	na		nl
9	-	-	-	-	-	-		↑ aCD16/56
10	-	-	-	-	-	na		↑ aCD3, ↑ aCD19, ↑ aCD16/56
11	-	-	+	-	-	na		↓ aCD3CD8, ↓ aCD19, ↑ aCD16/56
12	-	-	+	+	-	na		↑ aCD3, ↑ aCD3CD4, ↑ aCD19
13	+	+	+	-	-	na		↓ aCD3CD4, ↓ aCD19, ↓ aCD16/56, ↑ aCD3CD8
14	-	-	-	+	+	+	IgG2, IgG4	nl
15[b]	-	-	-	-	+	-		nl
16[c]	-	-	+	+	-	+	IgG1, IgG2, IgG4	↓ aCD3, ↓ aCD3CD4, ↓ aCD19, ↓ aCD16/56
17[d]	-	-	-	+*	-	+**	IgG2, IgG4	nl
18[e]	-	-	+	-	-	+	IgG1, IgG2, IgG4	↓ aCD19, ↓ aCD16/56
19[e]	-	-	+	+*	-	+	IgG1	na
20[d]	-	-	-	+*	-	+	IgG4	↓ aCD16/56
21	-	-	-	-	-	+	IgG3, IgG4	↓ aCD19 cells, slightly ↓ aMZ-like B, ↑ aCD3, ↑ aCD3CD8
22	-	+	+	+	+	-		↓ aCD3, ↓ aCD3CD4, ↓ aCD3CD8, ↓aCD19 cells (BM)
23[f]	-	-	+	+	-	+	IgG1, IgG2, IgG4	↓ aCD3CD4, ↓ aCD16/56
24[f]	-	-	+	+*	-	+***	IgG2, IgG3, IgG4	↓ aCD3CD8
25[g]	-	-	+	+	+	na		Na
26	-	-	+	-	+	+	IgG1, IgG3	Na
27	-	-	-	-	-	-		↓ aCD3, ↓ aCD3CD4, ↓ aCD3CD8, ↓ aCD19, ↓ aCD16/56
28	-	-	-	+	+	na		↑ aCD3, ↑ aCD3CD4, ↑ aCD3CD8, ↑ aCD19
29	-	-	-	-	-	-		↑ a CD3CD8, ↑ aCD19
30	-	-	-	-	+	na		↓ aCD3, ↓ aCD3CD8 cells, ↑ aCD19
31[h]	-	-	-	+	-	+	IgG2	↑ aCD3, ↑ aCD3CD4, ↑ aCD3CD8, ↑ aCD19
32	-	-	-	+	+	na		↑ aCD19
33	-	-	-	-	-	+	IgG1	Na
34	-	-	-	-	-	-		Nl
35	-	-	-	+	-	+	IgG2	Nl
36	-	-	+	+	-	na		Na
37	-	-	+	+	+	+	IgG1, IgG2	↓ aCD3, ↓ aCD3CD8, ↓ aCD19, ↓ aCD16/56 cells, ↓ a memB
38[i]	-	-	+	-	-	na		Na
39[i]	-	-	na	na	na	na		↑ aCD3CD4
40[i]	-	-	-	-	-	na		↑ aCD3, ↑ aCD3CD4
41[i]	-	-	-	-	-	na		Na
42[i]	-	-	-	-	-	-		↑ aCD3, ↑ aCD3CD4, ↑ aCD19, ↑ aCD16/56
43	-	-	+	+	-	+	IgG2	↑ aCD3, ↑ aCD3CD4, ↑ aCD3CD8, ↑ aCD 19
44	-	-	-	-	-	+	IgG1	Na
45	-	-	-	+	-	-		Na
46	-	-	+	+	+	na		Nl

Nr	Resp TV	Resp P	L function	G function
1	↓	↓	na	na
2[a]	nl	Nl	↓ NK toxicity In vitro lymphocyte proliferation: nl	Oxidative burst borderline ↓
3[a]	nl	Nl	↓ vitro lymphocyte proliferation decreased from 7 years on: SEA	Moderate ↓ oxidative burst
4	na	nl	na	na
5	na	na	In vitro lymphocyte proliferation: nl	na
6	nl	nl	na	nl
7	↓	na	na	na
8	na	na	na	na
9	↓	na	nl	na
10	nl	na	Thymic function: nl	na
11	na	nl	na	nl
12	nl	na	na	na
13	na	na	na	na
14	na	↓[j]	na	na
15[b]	na	na	nl	nl
16[c]	na	na	na	na
17[d]	na	na	na	na
18[e]	na	na	na	na
19[e]	na	na	na	na
20[d]	na	na	na	na
21	nl	nl	nl	na
22	nl	nl	na	na
23[f]	nl	nl [j]	↓ In vitro lymphocyte proliferation: PHA= 85%, PWD=72%, ConA=39%	na
24[f]	nl	nl [j]	↓ In vitro lymphocyte proliferation: PHA= 92%, PWD=87%, ConA=28%	na
25[g]	nl	↓	↓ In vitro lymphocyte proliferation: PHA	na
26	na	↓	na	na
27	↓	↓	↓ In vitro lymphocyte proliferation: PHA	na
28	nl	nl	na	nl
29	nl	↓	na	nl
30	nl	↓	na	nl
31[h]	nl	↓	na	nl
32	nl	nl	na	nl
33	na	nl	na	na
34	na	↓	na	nl
35	na	↓	na	na
36	na	na	na	na
37	na	na	na	na
38[i]	na	↓	na	na
39[i]	na	↓	na	na
40[i]	na	↓	na	na
41[i]	na	↓	na	na
42[i]	na	↓	na	na
43	nl	↓	na	na
44	na	na	na	na
45	na	na	na	na
46	na	na	na	na

Headings

Nr: patient number, NP: neutropenia, LP: lymphopenia,↓G: low IgG,↓A: low IgA,↓M: low IgM,↓IgG subclass: low IgG subclasses, Resp TV: response tetanus vaccine, Resp P: response PneumoVax® or Pneumo23®, L function: lymphocyte function tests, G function: granulocyte function tests.

Patients

(a) previously published in Seidel MG, Duerr C, Woutsas S, et al. J Med Genet 2014;51:254-263, (b) previously published in Celmeli F, J Investig Allergol Clin Immunol. 2014;24(6):442-4, (c) previously published in Seppänen et al. J Clin Immunol 2014;34:114–118., (d) family members and previously published in Dostal et al. International Journal of Immu-genetics 2007;34: 143–147 : patient 17 as IV:4 and patient 20 as IV, (e) family members, together with excluded patient 2, 3 and 4, (f) publication in press, Calvo Campoverde K, et al. Allergologia et Immunopathologia 2016, (g) previously published in Fernandez-San Jose C, J Paediatr Child Health 2011;47(7):485-6. (h) previously published in Browning MJ, J Investig Allergol Clin Immu-l 2010;20(3):263-266, (i) previously published in Keller MD, et al. Am J Med Genet C Semin Med Genet. 2013;163C(1):50-4, (j) decreased response to Pneumovax ® or Pneumo23® based on total IgG for S. pneumoniae.

* IgA completely absent, ** IgG2 completely absent, ***IgG3 completely absent.

Other abbreviations

a: absolute cell count, BM: bone marrow, CD: cluster of differentiation, ConA: Concanavalin A, memB: memory B cells, MZ: marginal zone, na: not available, nl: normal, PHA: phytohaemagglutinin, PWD: pokeweed mitogen, SEA: Staphylococcus aureus enterotoxin A, smB: switched memory B cells.

Conclusion

This retrospective survey demonstrates that patients with chromosomal aberrations and recurrent infections may harbor underlying primary immunodeficiencies. By specifically excluding the syndromic immunodeficiencies associated with Down and DiGeorge syndromes, we showed that a diverse spectrum of chromosomal aberrations can be associated with immunological abnormalities. In our cohort antibody deficiency was the most prevalent; this is important because infectious complications can be prevented with early interventions like antibiotic prophylaxis or immunoglobulin replacement treatment in these patients. To assess whether this association is a truly causal relation, a large case-control study would be needed; this is not really feasible. And of course, our survey results do not negate other contributing factors (e.g. aspiration; abnormal anatomy) in the development of recurrent ENT and airway infections in these patients. Nonetheless, our findings suggest it is important to consider immunological investigations in patients with chromosomal aberration and recurrent infections.

Acknowledgments
We would like to thank the Jeroen Bosch Hospital for providing the online survey system for free, Ms Riet Strik-Albers Nurse Practitioner (Amalia Children's Hospital, Radboud University Medical Center, Nijmegen, the Netherlands), Ms Nuria Murtra (Hospital Vall d'Hebron, Barcelona, Spain) and Ms Mélanie Langelier Research Nurse (B.Sc.N; Infectious Disease Susceptibility Program; McGill University Health Centre, Montreal, Canada) for help in data collection and the ESID Board for the permission to perform this study. Special thanks to The Focus Foundation for their continued support of rare disorders, particularly the X and Y chromosomal variation population.

References

1. Shima H, Kitagawa H, Wakisaka M, Furuta S, Hamano S, Aoba T: The usefulness of laryngotracheal separation in the treatment of severe motor and intellectual disabilities. Pediatr Surg Int 2010, 26(10):1041-4.
2. Yong PL, Boyle J, Ballow M, Boyle M, Berger M, Bleesing J, Bonilla FA, Chinen J, Cunninghamm-Rundles C, Fuleihan R *et al*: Use of intravenous immunoglobulin and adjunctive therapies in the treatment of primary immunodeficiencies: A working group report of and study by the Primary Immunodeficiency Committee of the American Academy of Allergy Asthma and Immunology. Clin Immunol, 135(2):255-63.
3. Quinti I, Soresina A, Guerra A, Rondelli R, Spadaro G, Agostini C, Milito C, Trombetta AC, Visentini M, Martini H *et al*: Effectiveness of immunoglobulin replacement therapy on clinical outcome in patients with primary antibody deficiencies: results from a multicenter prospective cohort study. J Clin Immunol 2011, 31(3):315-22.
4. Bousfiha A, Jeddane L, Al-Herz W, Ailal F, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, Etzioni A, Franco JL *et al*: The 2015 IUIS Phenotypic Classification for Primary Immunodeficiencies. J Clin Immunol 2015, 35(8):727-38.
5. Ming JE, Stiehm ER, Graham JM, Jr.: Syndromic immunodeficiencies: genetic syndromes associated with immune abnormalities. Crit Rev Clin Lab Sci 2003, 40(6):587-642.
6. Kusters MA, Verstegen RH, Gemen EF, de Vries E: Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol 2009, 156(2):189-93.
7. Davies EG: Immunodeficiency in DiGeorge Syndrome and Options for Treating Cases with Complete Athymia. Front Immunol 2013, 4(2013):322.
8. Lorini R, Ugazio AG, Cammareri V, Larizza D, Castellazzi AM, Brugo MA, Severi F: Immunoglobulin levels, T-cell markers, mitogen responsiveness and thymic hormone activity in Turner's syndrome. Thymus 1983, 5(2):61-6.
9. Hanley-Lopez J, Estabrooks LL, Stiehm R: Antibody deficiency in Wolf-Hirschhorn syndrome. J Pediatr 1998, 133(1):141-3.
10. Bart IY, Weemaes CM, Schuitema-Dijkstra AR, Smeets D, de Vries E: Immunodeficiency in a child with partial trisomy 6p. Acta Paediatr 2011, 100(8):e92-4.
11. Keller MD, Sadeghin T, Samango-Sprouse C, Orange JS: Immunodeficiency in patients with 49,XXXXY chromosomal variation. Am J Med Genet C Semin Med Genet 2013, 163C(1):50-4.
12. McGoey RR, Gedalia A, Marble M: Monosomy 18p and immunologic dysfunction: review of the literature and a new case report with thyroiditis, IgA deficiency, and systemic lupus erythematosus. Clin Dysmorphol 2011, 20(2):127-30.
13. Seidel MG, Duerr C, Woutsas S, Schwerin-Nagel A, Sadeghi K, Neesen J, Uhrig S, Santos-Valente E, Pickl WF, Schwinger W *et al*: A novel immunodeficiency syndrome associated with partial trisomy 19p13. J Med Genet 2014, 51(4):254-63.
14. Seppanen M, Koillinen H, Mustjoki S, Tomi M, Sullivan KE: Terminal deletion of 11q with significant late-onset combined immune deficiency. J Clin Immunol 2014, 34(1):114-8.
15. Recalcati MP, Valtorta E, Romitti L, Giardino D, Manfredini E, Vaccari R, Larizza L, Finelli P: Characterisation of complex chromosome 18p rearrangements in two syndromic patients with immunological deficits. Eur J Med Genet 2010, 53(4):186-91.
16. Batanian JR, Braddock SR, Christensen K, Knutsen AP: Combined immunodeficiency in a 3-year-old boy with 16p11.2 and 20p12.2-11.2 chromosomal duplications. Am J Med Genet A 2013, 164A(2):535-41.
17. Browning MJ: Specific polysaccharide antibody deficiency in chromosome 18p deletion syndrome and immunoglobulin A deficiency. J Investig Allergol Clin Immunol 2010, 20(3):263-6.
18. Balikova I, Vermeesch JR, Fryns JP, Van Esch H: Bronchiectasis and immune deficiency in an adult patient with deletion 2q37 due to an unbalanced translocation t(2;10). Eur J Med Genet 2009, 52(4):260-1.
19. Artac H, Reisli I, Yildirim MS, Bagci G, Luleci G, Hosgor O, Karaaslan S: Hypogammaglobulinemia and Silver-Russell phenotype associated with partial trisomy 7q and partial monosomy 21q. Am J Med Genet A 2009, 149A(2):277-9.

20. Dostal A, Linnankivi T, Somer M, Kahkonen M, Litzman J, Tienari P: Mapping susceptibility gene locus for IgA deficiency at del(18)(q22.3-q23); report of familial cryptic chromosome t(18q; 10p) translocations. Int J Immunogenet 2007, 34(3):143-7.
21. Cingoz S, Bisgaard AM, Bache I, Bryndorf T, Kirchoff M, Petersen W, Ropers HH, Maas N, Van Buggenhout G, Tommerup N *et al*: 4q35 deletion and 10p15 duplication associated with immunodeficiency. Am J Med Genet A 2006, 140(20):2231-5.
22. Sripanidkulchai R, Suphakunpinyo C, Jetsrisuparb C, Luengwattanawanich S: Thai girl with ring chromosome 18 (46XX, r18). J Med Assoc Thai 2006, 89(6):878-81.
23. Broides A, Ault BH, Arthus MF, Bichet DG, Conley ME: Severe combined immunodeficiency associated with nephrogenic diabetes insipidus and a deletion in the Xq28 region. Clin Immunol 2006, 120(2):147-55.
24. Imai K, Shimadzu M, Kubota T, Morio T, Matsunaga T, Park YD, Yoshioka A, Nonoyama S: Female hyper IgM syndrome type 1 with a chromosomal translocation disrupting CD40LG. Biochim Biophys Acta 2006, 1762(3):335-40.
25. Kellermayer R, Gyarmati J, Czako M, Teszas A, Masszi G, Ertl T, Kosztolanyi G: Mos 46,XX,r(18).ish r(18)(18ptel-,18qtel-)/46,XX.ish del(18)(18ptel-): an example for successive ring chromosome formation. Am J Med Genet A 2005, 139(3):234-5.
26. Fernandez-San Jose C, Martin-Nalda A, Vendrell Bayona T, Soler-Palacin P: Hypogammaglobulinemia in a 12-year-old patient with Jacobsen syndrome. J Paediatr Child Health 2011, 47(7):485-6.
27. Celmeli F, Turkkahraman D, Cetin Z, Mihci E, Yegin O: Selective IgM deficiency in a boy with ring chromosome 18. J Investig Allergol Clin Immunol, 24(6):442-4.
28. Mahmoud SA, Lowery-Nordberg M, Chen H, Thurmon T, Ursin S, Bahna SL: Immune defects in subjects with dysmorphic disorders. Allergy Asthma Proc 2005, 26(5):373-81.
29. de Vries E: Patient-centred screening for primary immunodeficiency, a multi-stage diagnostic protocol designed for non-immunologists: 2011 update. Clin Exp Immunol 2012, 167(1):108-19.
30. Schatorje EJ, Gemen EF, Driessen GJ, Leuvenink J, van Hout RW, van der Burg M, de Vries E: Age-matched reference values for B-lymphocyte subpopulations and CVID classifications in children. Scand J Immunol 2011, 74(5):502-10.
31. Schatorje EJ, Gemen EF, Driessen GJ, Leuvenink J, van Hout RW, de Vries E: Pediatric reference values for the peripheral T-cell compartment. Scand J Immunol 2012.
32. L.G. Shaffer JM-J, M. Schmid ISCN (2013): An International System for Human Cytogenetic Nomenclature. Basel: S. Karger; 2013.
33. Brodszki N, Jonsson G, Skattum L, Truedsson L: Primary immunodeficiency in infection-prone children in southern Sweden: occurrence, clinical characteristics and immunological findings. BMC immunology 2014, 15:31.
34. Al-Herz W, Bousfiha A, Casanova JL, Chatila T, Conley ME, Cunningham-Rundles C, Etzioni A, Franco JL, Gaspar HB, Holland SM *et al*: Primary immunodeficiency diseases: an update on the classification from the international union of immunological societies expert committee for primary immunodeficiency. Front Immunol 2011, 5:162.
35. Auphan N, DiDonato JA, Rosette C, Helmberg A, Karin M: Immunosuppression by glucocorticoids: inhibition of NF-kappa B activity through induction of I kappa B synthesis. Science 1995, 270(5234):286-90.
36. Berend SA, Shaffer LG, Bejjani BA: Pure trisomy 10p involving an isochromosome 10p. Clin Genet 1999, 55(5):367-71.
37. Lozic B, Culic V, Lasan R, Tomasovic M, Samija RK, Zemunik T: Complete trisomy 10p resulting from an extra stable telocentric chromosome. Am J Med Genet A 2012, 158A(7):1778-81.
38. Stone D, Ning Y, Guan XY, Kaiser-Kupfer M, Wynshaw-Boris A, Biesecker L: Characterization of familial partial 10p trisomy by chromosomal microdissection, FISH, and microsatellite dosage analysis. Hum Genet 1996, 98(4):396-402.
39. Slyper AH, Pietryga D: Conversion of selective IgA deficiency to common variable immunodeficiency in an adolescent female with 18q deletion syndrome. Eur J Pediatr 1997, 156(2):155-6.

40. Rosen P, Hopkin RJ, Glass DN, Graham TB: Another patient with chromosome 18 deletion syndrome and juvenile rheumatoid arthritis. J Rheumatol 2004, 31(5):998-1000.
41. Gordon MF, Bressman S, Brin MF, de Leon D, Warburton D, Yeboa K, Fahn S: Dystonia in a patient with deletion of 18q. Mov Disord 1995, 10(4):496-9.
42. Lipschutz W, Cadranel S, Lipschutz B, Martin L, Clees N, Martin JJ, Wauters JG, Coucke P, Willems P: 18q-syndrome with coeliac disease. Eur J Pediatr 1999, 158(6):528.
43. Schaub RL, Hale DE, Rose SR, Leach RJ, Cody JD: The spectrum of thyroid abnormalities in individuals with 18q deletions. J Clin Endocrinol Metab 2005, 90(4):2259-63.
44. Mattina T, Perrotta CS, Grossfeld P: Jacobsen syndrome. Orphanet J Rare Dis 2009, 4:9.
45. Penny LA, Dell'Aquila M, Jones MC, Bergoffen J, Cunniff C, Fryns JP, Grace E, Graham JM, Jr., Kousseff B, Mattina T *et al*: Clinical and molecular characterization of patients with distal 11q deletions. Am J Hum Genet 1995, 56(3):676-83.
46. von Bubnoff D, Kreiss-Nachtsheim M, Novak N, Engels E, Engels H, Behrend C, Propping P, de la Salle H, Bieber T: Primary immunodeficiency in combination with transverse upper limb defect and anal atresia in a 34-year-old patient with Jacobsen syndrome. Am J Med Genet A 2004, 126A(3):293-8.
47. Miller DT NR, Sobeih MM, et al.: 16p11.2 Microdeletion. In. Edited by Pagon RA AM, Ardinger HH, et al.: GeneReviews® [Internet]. Seattle (WA): University of Washington, Seattle; 1993-2015. Available from: http://www.ncbi.nlm.nih.gov/books/NBK11167/; 2009 Sep 22 [Updated 2011 Oct 27]. .
48. Maggadottir SM, Li J, Glessner JT, Li YR, Wei Z, Chang X, Mentch FD, Thomas KA, Kim CE, Zhao Y *et al*: Rare variants at 16p11.2 are associated with common variable immunodeficiency. J Allergy Clin Immunol 2015, 135(6):1569-77.
49. Shiow LR, Paris K, Akana MC, Cyster JG, Sorensen RU, Puck JM: Severe combined immunodeficiency (SCID) and attention deficit hyperactivity disorder (ADHD) associated with a Coronin-1A mutation and a chromosome 16p11.2 deletion. Clin Immunol 2009, 131(1):24-30.
50. Foger N, Rangell L, Danilenko DM, Chan AC: Requirement for coronin 1 in T-lymphocyte trafficking and cellular homeostasis. Science 2006, 313(5788):839-42.

Additional file 1: the Online Questionnaire

I. ***Physician*** *submitting the survey (one form per submitted patient).* NB the reporting physician, not the patient!
This information will be used as author information for the manuscript.

1. family name ...
2. initials ...
3. titles ...
4. affiliation ...
5. email address ...

II. ***Patient*** *with chromosomal aberration and associated immunological abnormalities being reported*

1. male/female
2. date of birth
3. date of report (today)
4. chromosomal aberration present not being Down syndrome (trisomy 21) or DiGeorge syndrome (22q11 deletion): yes/no
5. please enter as full a description of the chromosomal aberration as possible (example: 46,XY,dup(6)(p12.2p21.31) or when SNP array was performed please state brand and type of chip used and SNP positions: example: Affymetrix 250k SNP 46,XX,der(2)t(2;10)(q37.3;q26.3)mat.arr snp 2q37.2q37.3(SNP_A-1957498->SNP_A-2027809) x1,10q26.3(SNP_A-2264115->SNP_A-1934598)x3): .....

III. Clinical characteristics of the reported ***patient***

1. Which clinical presentations apply to the patient? (multiple answers possible)

a. Recurrent ENT and airway infections
b. Failure to thrive from early infancy
c. Recurrent pyogenic infections
d. Unusual infections or unusually severe course of infections
e. Recurrent infections with the same type of pathogen
f. Autoimmune or chronic inflammatory disease; lymphoproliferation

2. What is the **clinically most important** clinical presentation of the patient? (single answer):

a. Recurrent ENT and airway infections
b. Failure to thrive from early infancy
c. Recurrent pyogenic infections
d. Unusual infections or unusually severe course of infections
e. Recurrent infections with the same type of pathogen
f. Autoimmune or chronic inflammatory disease; lymphoproliferation

3. Does the patient suffer from (multiple answers possible):

a. developmental delay
b. ataxia, paresis or other motor disability
c. dysmorphic features
d. microcephaly
e. growth retardation
f. atopic eczema
g. hair and/or nail abnormalities
h. hypopigmentation

4. Any other relevant clinical information, specification of the above:

IV. Immunological characteristics of the reported ***patient***

1. Was a leukocyte differential performed (absolute numbers)? yes/no
2. if yes: granulocytes (10e9/l)
3. if yes: lymphocytes (10e9/l)
4. Were immunoglobulins (IgG, IgA, IgM) determined in serum? yes/no
5. if yes: level of IgG (g/l)
6. if yes: level of IgA (g/l)
7. if yes: level of IgM (g/l)
8. Were IgG-subclasses determined in serum? yes/no
9. if yes: level of IgG1 (g/l)
10. if yes: level of IgG2 (g/l)
11. if yes: level of IgG3 (g/l)
12. if yes: level of IgG4 (g/l)
13. Were lymphocyte subpopulations determined?
no / yes (percentage only) / yes (absolute number) (single answer)

14. if yes (%): $CD3^+$ T-lymphocytes
15. if yes (%): $CD3^+CD4^+$ helper-T-lymphocytes
16. if yes (%): $CD3^+CD8^+$ cytotoxic T-lymphocytes
17. if yes (%): $CD19^+$ or $CD20^+$ B-lymphocytes
18. if yes (%): $CD3^-$ CD16 and/or $CD56^+$ NK-cells
19. if yes (10e9/l): $CD3^+$ T-lymphocytes
20. if yes (10e9/l): $CD3^+CD4^+$ helper T-lymphocytes
21. if yes (10e9/l): $CD3^+CD8^+$ cytotoxic T-lymphocytes
22. if yes (10e9/l): $CD19^+$ or $CD20^+$ B-lymphocytes
23. if yes (10e9/l): $CD3^-$ CD16 and/or $CD56^+$ NK-cells
24. Were any other lymphocyte subpopulations determined? yes/no.
if yes: please email or fax an anonymized copy of the results
25. Were vaccine response(s) determined? no / yes (tetanus) / yes (PneumoVax®, Pneumo23®) / yes (other) (multiple answer)
if yes: please email or fax an anonymized copy of the results
26. Were any tests of granulocyte function performed? yes/no
if yes: please email or fax an anonymized copy of the results
28. Were any tests of lymphocyte function performed? yes/no
if yes: please email or fax an anonymized copy of the results

Thank you for returning this online survey!

Additional File 2. Clinical and immunological characteristics of the excluded patients.

Part A. Clinical characteristics of the excluded patients.

Nr	Sex	Age (yrs)[1]	Genetics	Immunological presentation[2]	Other clinical presentations[3]	Other symptoms
Excl 1	M	39.4	Rubinstein Taybi Syndrome (mutation in CREBBP gene)	Airways	Developmental delay Dysmorphic features Microcephaly Growth retardation	
Excl 2*	F	41.7	46,XX,t(12;14)(p11.2;q13)	Airways	Atopic eczema	Atopy, eczema, asthma, allergy and angioedema; no immunodeficiency
Excl 3*	F	16.8	46,XX,t(12;14)(p11.2;q13)	Airways	Atopic eczema	Atopy, asthma, allergy and anaphylaxis; no immunodeficiency
Excl 4*	F	13.7	46,XX,t(12;14)(p11.2;q13)	AI disease	Atopic eczema	ALL, atopy, asthma and allergy; no immunodeficiency
Excl 5	F	14.8	Kabuki syndrome (no genetic diagnosis)	Airways	Developmental delay Dysmorphic features Growth retardation	
Excl 6	M	4.6	Rothmund Thomson Syndrome (mutation in RECQL4 gene)	Unusual infections	Dysmorphic features Growth retardation Atopic eczema	

Headings
Nr= patient number; [1]at the time of reporting; [2]most prominent immunological clinical presentation; [3]other clinical presentations as requested in the survey (Additional File 1).
Clinical presentations
Airways = Recurrent ENT and airway infections; unusual infections = unusual infections or unusually severe course of infections; AI disease = autoimmune or chronic inflammatory disease; lymphoproliferation (de Vries 2012).
Patients
* family members together with included patients 18 and 19
Other abbreviations
ALL: acute lymphatic leukemia, Excl: excluded patient,F: female, M: male, yrs: years.

Part B. Immunological characteristics of the excluded patients.

Nr	NP	LP	↓G	↓A	↓M	↓IgG subclass	Lymphocyte subsets	Resp TV	Resp P	L function	G function
Excl 1	-	-	+	+	+	na	↓ aCD16/56	na	na	na	na
Excl 2*	-	-	-	-	-	-	na	na	na	na	na
Excl 3*	-	-	-	-	-	-	na	na	na	na	na
Excl 4*	-	-	-	-	-	-	na	na	na	na	na
Excl 5	-	-	+	+	-	+ IgG1, IgG2, IgG4	↑ aCD3 cells, ↓ a sm and MZ B, ↑ CD21low B	nl	nl**	na	na
Excl 6	-	-	+	+	-	na	↓ aCD3, ↓aCD3CD4, ↓ aCD3CD8, ↓ aCD19, ↓ aCD16/56 cells	na	na	In vitro lymphocyte proliferation decreased: PHA, PWM, OKT3	na

Headings

Nr: patient number, NP: neutropenia, LP: lymphopenia, ↓G: low IgG, ↓A: low IgA, ↓M: low IgM. ↓IgG subclass: low IgG subclasses, Resp TV: response tetanus vaccine, Resp P: response PneumoVax® or Pneumo23®, L function: lymphocyte function tests, G function: granulocyte function tests.

Patients

* family members together with included patients 18 and 19, ** decreased response to Pneumovax® or Pneumo23® based on total IgG for S. pneumoniae.

Other abbreviations

a: absolute count, CD: cluster of differentiation, MZ: marginal zone, na: not available, nl: normal, PHA: phytohaemagglutinin, PWM: pokeweed mitogen, OKT3: a monoclonal IgG2 antibody which binds the ε component of the CD3 signal-transduction complex.

Additional File 3.

Additional test results of the included patients.

Nr	Lymphocyte subpopulations (x10⁶9/l)	Additional immunological/ hematological findings		Other laboratory	MRI
1	CD3 2.19, CD3 1.70, CD8 0.52, CD19 0.50, CD16/56 0.15	na		na	na
2[a]	CD3 1.21, CD4 1.70, CD8 0.20, CD19 0.77, CD16/56 0.16	NK degranulation:↓	MBL ↓; CH50/AH50 nl	Alpha-Thalassemia Trait	Multiple diffuse hyperintense white matter lesions, pronounced atrophy, hydrocephalus, microcysts basal ganglia
3[a]	CD3 3.78, CD4 1.85, CD8 1.43, CD19 2.64, CD16/56 0.29	NK degranulation: ↓	MBL ↓; CH50/AH50 nl	na	Hypoplastic inferior vermis, sligthly extended temporal lobe
4	na	na		na	na
5	na	na		na	na
6	CD3 3.05, CD4 1.91, CD8 0.94, CD19 1.97, CD16/56 0.40	na		na	na
7	CD3 1.03, CD4 0.72, CD8 0.32, CD19 0.29, CD16/56 0.10	Auto-immune anemia		na	na
8	CD3 4.09, CD4 2.76, CD8 1.26, CD19 0.58, CD16/56 0.46	na		na	na
9	CD3 1.70, CD4 0.93, CD8 0.54, CD19 0.46, CD16/56 0.74	Hypergammapathy, pernicious anemia		na	na
10	CD3 5.60, CD4 3.20, CD8 2.00, CD19 3.40, CD16/56 0.63	Lymphocytosis		na	na
11	CD3 0.74, CD4 0.56, CD8 0.16, CD19 0.10, CD16/56 0.44	na		na	na
12	CD3 3.69, CD4 2.78, CD8 0.91, CD19 1.48, CD16/56 0.34	na		na	na
13	CD3 2.26, CD4 0.43, CD8 1.80, CD19 0.05, CD16/56 0.02	Monocytopenia		na	na
14	CD3 0.99, CD4 0.46, CD8 0.41, CD19 0.24, CD16/56 0.11	na		na	na
15[b]	na	Isohemagglutinin titers 1:1		na	Corpus callosum hypoplasia, delayed myelinisation
16[c]	CD3 0.37, CD4 0.09, CD8 0.27, CD19 0.02, CD16/56 0.05	Chronic Paris-Trousseau type thrombocytopenia		na	na
17[d]	CD3 0.80, CD4 0.51, CD8 0.02, CD19 0.29, CD16/56 0.16	na		na	na
18[e]	CD3 1.41, CD4 0.89, CD8 0.42, CD19 0.14, CD16/56 0.09	na		na	Occult subarachnoidal cyst left frontal lobe
19[e]	na	na		na	na
20[d]	CD3 0.96, CD4 0.67, CD8 0.28, CD19 0.24, CD16/56 0.07	na		na	Demyelinating white matter lesions
21	CD3 1.94, CD4 1.10, CD8 0.97, CD19 0.15, CD16/56 0.28	na		Hypergonadotropic hypogonadism Primary hypophosphatasia	na
22	CD3 0.60, CD4 0.33, CD8 0.16, CD19 0.07, CD16/56 0.12	Thrombocytopenia		na	na
23[f]	CD3 2.16, CD4 1.20, CD8 0.80, CD19 0.53, CD16/56 0.12	na		na	Hypoplasia corpus callosum, ventriculomegaly
24[g]	CD3 1.16, CD4 0.80, CD8 0.26, CD19 0.26, CD16/56 0.26	na		na	na
25[g]	CD3 0.72, CD4 0.46, CD8 0.21, CD19 0.06, CD 16/56 0.07	Thrombocytopenia		na	na
26	na	na		na	na

Additional File 3 continued

Nr	Lymphocyte subpopulations (x10⁶9/l)	Additional immunological/ hematological findings	Other laboratory	MRI
27	CD3 0.53, CD4 0.25, CD8 0.27, CD19 0.11, CD16/56 0.09	na	na	na
28	CD3 3.08, CD4 1.98, CD8 1.15, CD19 0.91, CD16/56 0.31	na	na	na
29	CD3 1.96, CD4 1.08, CD8 0.82, CD19 0.59, CD16/56 0.32	na	na	na
30	CD3 0.86, CD4 0.61, CD8 0.22, CD19 0.84, CD16/56 0.24	na	na	na
31[h]	CD3 2.57, CD4 1.35, CD8 1.11, CD19 0.66, CD16/56 0.15	na	na	Low myelinisation
32	CD3 2.79, CD4 1.66, CD8 1.00, CD19 0.77, CD16/56 0.21	na	na	na
33	na	na	na	na
34	na	na	na	na
35	na	na	na	na
36	na	na	na	na
37	na	na	na	na
38[i]	na	na	na	na
39[i]	CD3 1.97, CD4 1.50, CD8 0.37, CD19 0.35, CD16/56 0.22	na	na	na
40[i]	CD 3 2.68, CD4 1.93, CD8 0.66, CD19 0.60, CD16/56 0.78	na	na	na
41[i]	na	na	na	na
42[i]	CD3 22.70, CD4 15.60, CD8 0.65, CD19 3.97, CD16/56 1.70	na	na	na
43	CD3 2.61, CD4 1.57, CD8 0.87, CD19 1.26, CD16/56 0.35	na	na	na
44	na	na	na	na
45	CD3 1.70, CD4 0.80, CD8 0.80, CD19 0.20, CD16/56 0.55	na	na	na
46	CD3 1.35, CD4 0.81, CD8 0.54, CD19 0.45, CD16/56 0.18	na	na	na

Patients

(a) previously published in Seidel MG, Duerr C, Woutsas S, et al. J Med Genet 2014;51:254-263, (b) previously published in Celmeli F, J Investig Allergol Clin Immunol. 2014;24(6):442-4, (c) previously publised in Seppänen et al. J Clin Immunol 2014;34:114–118., (d) family members and previously published in Dostal et al. International Journal of Immu-genetics 2007;34: 143–147 : patient 17 as IV:4 and patient 20 as IV, (e) family members, together with excluded patient 2, 3 and 4, (f) publication in press, Calvo Campoverde K, et al. Allergologia et Immunopathologia 2016, (g) previously published in Fernandez-San Jose C, J Paediatr Child Health 2011;47(7):485-6. (h) previously published in Browning MJ, J Investig Allergol Clin Immu-l 2010;20(3):263-266, (i) previously published in Keller MD, et al. Am J Med Genet C Semin Med Genet. 2013;163C(1):50-4.

Other abbrevations

AH: alternative complement, CD: cluster of differentiation, CH: classical complement, MBL: mannose binding ligand, na: not available, nl: normal.

DISCUSSION AND FUTURE PERSPECTIVES

Diagnosing primary immunodeficiency (PID) in children is challenging. On the one hand, this is due to the maturing immune system which makes it difficult to distinguish between a physiologically immature and a pathologically dysfunctional immune system. On the other hand, it is also due to the unawareness of doctors. The aim of this thesis was (1) to get more insight into the pitfalls of the developing immune system for doctors confronted by children with potential primary immunodeficiency and (2) to study in detail the clinical picture of hypogammaglobulinemia in children using data from (inter)national registries and surveys. Our age-matched reference values for lymphocyte subpopulations presented in this thesis help to distinguish between normal and abnormal results. Furthermore, we show that analysis of larger patient cohorts, using registries and/or surveys, can detect unknown patterns, like the predominance of boys with hypogammaglobulinemia. These findings can give new impulse to future research.

Pediatric immune reference values for diagnosis and classification

Predominantly antibody disorders (PAD), the most common forms of PID, result from either quantitative or qualitative/functional cellular deficits. For diagnosis principally antibody concentrations are used and compared to age specific references values. In addition functional responses to vaccinations and natural infections help making a diagnosis. As PAD are a heterogeneous group of disorders, further classifications have been developed relating to molecular, cellular development or functional deficits. Most of these classifications were developed for CVID patients, it is important to realise however that the diagnostic criteria for CVID used by the various authors have varied with time (and opinion).

At first, flow cytometric classification schemes of common variable immunodeficiency disorders (CVID) were developed in small patient groups [1, 2]. In 2008 a large European trial was published, proposing a B cell phenotypic classification of a large cohort of CVID patients: the EUROclass trial [3]. This study, including mainly adult patients, showed that a severe reduction of switched memory B cells was associated with a higher risk for splenomegaly and granulomatous disease. An expansion of $CD21^{low}$ B cells marked patients with splenomegaly, and lymphadenopathy was significantly linked with transitional B cell expansion. There are only a few studies looking at specific classification schemes in pediatric CVID patients [4, 5]. Because of their maturing immune system, classification schemes developed in adult CVID patients, cannot simply be extrapolated to use in children. One study showed that there is a relation between decreased class-switched memory B cells in combination with increased transitional B cells and the occurrence of disease related complications. In this pediatric CVID group, no correlation between clinical characteristics and T cell phenotype could be made [4]. Another study looking at peripheral B cell analysis in pediatric CVID patients showed that patients with a block in the early B cell maturation phase require earlier immunoglobulin replacement therapy and that these patients were at significantly greater risk of enteropathy,

granuloma formation, cytopenia, and lymphoproliferation. B cell maturation inhibited at the natural effector stage was associated with higher risk of autoimmune manifestations other than autoimmune cytopenia (i.e. arthritis, auto-immune thyroiditis and auto-immune hepatitis) [5].

Subsequently, a classification of CVID based on clinical phenotypes was proposed [6]. Five distinct clinical phenotypes were defined: no complications, autoimmunity, polyclonal lymphocytic infiltration, enteropathy and lymphoid malignancy. A total of 83% of included patients had only one of these phenotypes. Higher serum IgM and lower circulating CD8 proportions were found to be predictive markers for polyclonal lymphocytic infiltration and autoimmunity respectively, but further there was a widespread variation in immunoglobulin levels and B cell numbers among the different clinical phenotypes. A pediatric CVID cohort showed that children fitting into one of the above mentioned clinical phenotypes were more affected in their numbers of class switched memory B cells than children without any disease related complications [4].

Others looked at the functional defects and described five different B cell patterns in CVID patients [7]. These five patterns give a good overview of possible defects in the late B cell development that can cause hypogammaglobulinemia: 1) B cell production and germinal center defect, 2) early peripheral B cell maturation or survival defect, 3) B cell activation and proliferation defect, 4) germinal center defect and 5) post germinal center defect. Splenomegaly significantly clustered in patients with B cell pattern 1 (B cell production and germinal center defect).

To determine whether the above described classification schemes based on or correlated with peripheral lymphocyte counts and all developed with mainly adult data, are applicable in children, reliable age-matched reference values are needed. This thesis provides these pediatric references values for B- and T-lymphocyte subpopulations in *chapter 1* and *chapter 2*, including also newly described lymphocyte subpopulations. These reference values are displayed as means with a range per specific age group, giving a 95% chance that 90% of healthy children will show numbers within this range. We show that the greatest changes in the peripheral B cell and T cell compartment occur in the first years of life, reflecting immune maturation. For B cells the increase in the first months of life is almost entirely caused by expansion of the naive B-lymphocyte pool, and to a small extent by expansion of transitional cells. The increase in T cells in the first 9-15 months of life is mainly caused by expansion of recent thymic emigrants and naive T cells. Regulatory T cells increase only in the first 5 months of life and then gradually decrease, whereas $CXCR5^+$ memory cells are virtually absent at birth and rapidly increase in the first months of life. These changes in the peripheral lymphocyte population are reflected best when using absolute numbers of cells, relative numbers can be misleading. Therefore, absolute B and T cell numbers should be used for diagnosis and research in PID [8].

Furthermore we tested the existing CVID classification schemes for applicability in children. In our study, 40 out of 97 healthy children would be classified in one of the EUROclass CVID subgroups due to the fact that they show lower relative numbers of

switched memory B-lymphocytes. The low relative numbers of switched memory B-lymphocytes in these healthy children are not a sign of immunodeficiency, but a reflection of physiological immune maturation. Although this mainly concerns young children below two years of age (38 out of 40), this illustrates that one should be careful when using adult flow cytometry classification schemes in children. In the functional classification scheme [7], patients are not only divided based on quantitative B cell numbers, but also on B cell replication history and SHM levels. Pattern 3 (B cell activation and proliferation defect) showed decreased proliferation of marginal zone like B cells with reduced SHM levels in combination with a reduction of marginal zone like and memory B cells. This thesis shows in *chapter 3* that SHM levels are also age dependent and seem to increase in the first two years of life. This can not only be explained by increase in mature B cells in these children. We hypothesize that increase of SHM in childhood reflects an increase of proportion of memory B cells originating from secondary GC-dependent B cell responses as well as increase of the SHM frequency within the memory B cell compartment, reflecting immune maturation. Finally, we show that age is the primary determinant of TACI expression on B cells, suggesting that decreased TACI expression on B cells in young children is not automatically indicative of a potential TACI mutation.

Insights from analysis of registry and survey cohorts

Male predominance PAD suggests hidden X-linked inheritance

The pathogenesis of PAD is still largely unclear, only in agammaglobulinemia and class switch recombination problems several gene defects have been identified. With the hallmark of hypogammaglobulinemia, the common immune defect is a loss of B cell function, but the causes of these abnormalities remain largely unknown. Several autosomal localized genetic defects in CVID patients have been discovered lately (i.e. ICOS [9], TACI [10], BAFF-R [11], lipopolysaccharide responsive beige-like anchor protein (LRBA) [12], phospholipase Cγ2 (PLCG2) [13], protein C kinase δ (PRKCD) [14], CD19 [15], CD20 [16], CD21 [17], CD81 [18] and NF-κB1 Subunit p50 [19]).

But in more than 95% of CVID patients the genetic background is unknown [20] and even less is known as yet about the genetic defects in primary hypogammaglobulinemias other than CVID. A genome-wide association study (GWAS) using single nucleotide polymorphism (SNP) arrays in a large cohort of CVID patients showed associations with the MHC region, the metalloproteinases ADAM28, ADAM7, ADAMDEC1 and STC1 [21]. Another GWAS study of IgAD patients from Sweden and Iceland identified an association with a variant in IFIH1 and CLEC16A, both known to be associated with autoimmune disease, as well as associations with class II alleles in the HLA region [22]. Furthermore, copy number variants association analysis uncovered several novel genes that were significantly associated with CVID [21].

Our studies suggest that X-linked inheritance may play a role in the development of primary hypogammaglobulinemia. In both clinical studies described in *Part 2* of this thesis boys predominate in the cohorts. In the hypogammaglobulinemia category of the ESID online database 63% were boys (*chapter 4*), and in the Dutch cohort of children with IgG-subclass deficiency and/or SPAD 67% were boys (*chapter 5*). Also, in the ESID registry boys were younger at diagnosis (mean age males 5.3, females 5.8 years) and suffered from more complications (12% in boys, 5% in girls). In the Dutch cohort, only boys showed progressive immunodeficiency during follow-up. This predominance of males can be found in other papers [3, 23-26] but was generally not given much attention. We hypothesize that this male predominance can be due to either insufficient diagnostic procedures or atypical presentation (e.g. a known X-linked PID diagnosis has been missed in both cases), or to unknown X-linked disease(s) that cause primary hypogammaglobulinemia.

IgG subclass deficiency and/or SPAD are identified in the majority by pediatric immunologist – do we miss patients in the general pediatric population?

As PIDs are rare, the awareness of PID among health care professionals is low; most professionals are not familiar with the details of these complex diseases. A recent study in Brazil among 4026 physicians (40% pediatricians) revealed that only 40% performed immunological screening in patients with frequent use of antibiotics. 77% of the doctors were not familiar with the PID warning signs [27]. The majority of children with recurrent infections however will present to a general pediatrician. Little is known about the prevalence, clinical presentation and prognosis of 'milder' forms of PAD, such as IgG-subclass deficiency and/or SPAD in children in the general pediatric population. The study described in *chapter 5* of this thesis was the first study published in a Western-European cohort of children with IgG-subclass deficiency and/or SPAD, and the first that included children followed outside tertiary centers [28-31]. We show that the most prevalent IgG-subclass deficiency in our cohort was IgG2 deficiency. This is in contrast with other published cohorts, where IgG3 deficiency was most common [28, 30]. These cohorts contained a higher number of children on prophylactic antibiotics, and were performed in tertiary populations. This may explain this difference in phenotype. Moreover, the majority of children (69%) in our cohort were reported by four secondary hospitals with a pediatric immunologist in the staff, which raises the question whether many of these patients are missed in the general pediatric population in the Netherlands. Although often considered to be mild, IgG2 deficiency and SPAD are risk factors for invasive pneumococcal disease [32], so awareness of these diseases is important.

PAD in chromosomal syndromic disorders – Importance of partial chromosomal monosomies and trisomies as hints for relevant genetic loci

Patients with chromosomal syndromic disorders often have recurrent infections, but physicians can easily ascribe these infections to other factors such as food and saliva aspiration, structural abnormalities of the upper respiratory tract, neuromuscular

problems, malnutrition or institutionalization [33]. Not always immunological workup is performed. However, several chromosomal disorders are known to be associated with PID. Down syndrome (trisomy 21) [34] and DiGeorge syndrome (22q11 deletion) [35] are well-known examples, but not the only ones. Our survey among ESID members (*chapter 6*) showed the association of a wide range of other chromosomal aberrations with immunological abnormalities. Up to one third of patients with chromosomal aberrations (defined as a missing, extra, or irregular portion [i.e. involving more than one gene] of chromosomal DNA) and recurrent infections may have some form of primary immunodeficiency. Antibody deficiency was the most prevalent in our cohort, which means that it might be possible to prevent infectious complications with antibiotic prophylaxis or immunoglobulin substitution in these patients. This is an important awareness message for pediatricians treating these patients. We could not identify a specific suspicious chromosome or region, however the described partial chromosomal monosomies and trisomies can serve as hints for relevant genetic loci in future studies.

Future perspective

Unravelling the genetic background of primary hypogammaglobulinemia will require further research in large patient cohorts. When 'using' patients with chromosomal aberrations for further research, it would be interesting to perform case-control studies, to assess whether various chromosomal aberrations are causally related to immunodeficiency and give us specific gene regions to explore and maybe target in the future. Furthermore, a search for known (i.e. BtK deficiency, CD40L deficiency and X-linked hypogammaglobulinemia and isolated growth hormone deficiency [XLH-GHD]) and unknown X-linked genetic defects in boys with complicated and early onset primary hypogammaglobulinemia could be very interesting as well. Exome sequencing of the X-chromosome in this population could be a suggestion.

But should we invest in knowing all these genetic details if therapy of hypogammaglobulinemia is simply prophylactic antibiotics and immunoglobulin substitution? This treatment is indeed effective in prevention of lower respiratory tract infections, but the effect of immunoglobulin substitution on non-infectious comorbidities (e.g. autoimmunity, lymphocytic hyperplasia and enteropathy) is doubtful [36-38]. More insight in the genetics and pathogenesis could also provide new treatment options for these non-infectious comorbidities. A good example are the recently discovered heterozygous loss-of-function mutations in CTLA-4 protein in patients with hypogammaglobulinemia, recurrent infections and multiple autoimmune features [39]. CTLA-4 is an essential negative regulator of immune responses by inhibiting CD28 T cell - B cell co-stimulation and loss-of-function mutations of CTLA-4 can therefore play an important role in developing multiple autoimmune features. Treatment with soluble CTLA-4 fusion proteins (i.e. Abatacept and Belatacept) could potentially be beneficial in the context of CTLA-4 deficiency. Another consequence could be the awareness of yet

unknown complications in the future, like the risk of vasculopathy in deficiency of Adenosine Deaminase 2 (ADA2) in patients with antibody deficiency and immune dysregulation [40]. Identification of single gene disease could even raise the possibility of gene therapy, like it is used nowadays in certain severe combined immunodeficiencies [41]. Of course this would only be suitable for severely affected patients, or for patients with a bad prognosis. This last patient category could benefit from early genetic diagnosis. Next to the genetic background of PAD, it would also be good to know which hypogammaglobulinemic children are at risk for developing more severe disease and thus need special attention. It is known that in children presenting with hypogammaglobulinemia, low IgM values between 2 and 5 years of age, an impaired antibody response as well as low B cell counts seem to be a risk factor for developing persistent hypogammaglobulinemia [42]. Recently it was also shown that a decreased IgA response to pneumococcal polysaccharides indicates persistent disease in children with hypogammaglobulinemia [43], and that absent or low numbers of switched memory B cells was found to be associated with a higher frequency of pneumonia and bronchiectasis in children with early onset hypogammaglobulinemia (defined as onset before the age of 6 years) [44]. To assess whether these are real risk factor for persisting and/or progressive hypogammaglobulinemia in children, a large prospective cohort study has to be performed. Online registries, like the ESID online database, should preferably be used to set up such a large international prospective trial. Automatic inferfaces transferring the patient data from the hospital file to specific registries, standardization of ethical procedures in Europe for using patient data for international registries and complex automated data quality checks in the registries are needed to make such trials feasible and worthwhile.

Final conclusions of the thesis

1. The greatest changes in the peripheral B cell and T cell compartment occur in the first years of life, reflecting immune maturation.
2. PID classification schemes developed with adult data cannot be used in children.
3. The increase of SHM in the first years of life can not only be explained by increase in numbers of mature B cells.
4. The male predominance in PAD patients suggests hidden X-linked inheritance.
5. Milder PAD, i.e. IgG-subclass deficiency and/or SPAD, are often missed in general pediatric care.
6. PID (mostly PAD) can also be a cause of recurrent infections in patients with chromosomal aberrations, next to other known risk factors.

Literature

1. Warnatz K, Denz A, Drager R, Braun M, Groth C, Wolff-Vorbeck G, Eibel H, Schlesier M, Peter HH: Severe deficiency of switched memory B cells (CD27(+)IgM(-)IgD(-)) in subgroups of patients with common variable immunodeficiency: a new approach to classify a heterogeneous disease. Blood 2002, 99(5):1544-51.
2. Piqueras B, Lavenu-Bombled C, Galicier L, Bergeron-van der Cruyssen F, Mouthon L, Chevret S, Debre P, Schmitt C, Oksenhendler E: Common variable immunodeficiency patient classification based on impaired B cell memory differentiation correlates with clinical aspects. J Clin Immunol 2003, 23(5):385-400.
3. Wehr C, Kivioja T, Schmitt C, Ferry B, Witte T, Eren E, Vlkova M, Hernandez M, Detkova D, Bos PR *et al*: The EUROclass trial: defining subgroups in common variable immunodeficiency. Blood 2008, 111(1):77-85.
4. van de Ven AA, van de Corput L, van Tilburg CM, Tesselaar K, van Gent R, Sanders EA, Boes M, Bloem AC, van Montfrans JM: Lymphocyte characteristics in children with common variable immunodeficiency. Clin Immunol 2010, 135(1):63-71.
5. Piatosa B, Pac M, Siewiera K, Pietrucha B, Klaudel-Dreszler M, Heropolitanska-Pliszka E, Wolska-Kusnierz B, Dmenska H, Gregorek H, Sokolnicka I *et al*: Common variable immune deficiency in children--clinical characteristics varies depending on defect in peripheral B cell maturation. J Clin Immunol 2013, 33(4):731-41.
6. Chapel H, Lucas M, Lee M, Bjorkander J, Webster D, Grimbacher B, Fieschi C, Thon V, Abedi MR, Hammarstrom L: Common variable immunodeficiency disorders: division into distinct clinical phenotypes. Blood 2008, 112(2):277-86.
7. Driessen GJ, van Zelm MC, van Hagen PM, Hartwig NG, Trip M, Warris A, de Vries E, Barendregt BH, Pico I, Hop W *et al*: B-cell replication history and somatic hypermutation status identify distinct pathophysiologic backgrounds in common variable immunodeficiency. Blood 2011, 118(26):6814-23.
8. de Vries E, de Bruin-Versteeg S, Comans-Bitter WM, de Groot R, Hop WC, Boerma GJ, Lotgering FK, Sauer PJ, van Dongen JJ: Neonatal blood lymphocyte subpopulations: a different perspective when using absolute counts. Biol Neonate 2000, 77(4):230-5.
9. Grimbacher B, Hutloff A, Schlesier M, Glocker E, Warnatz K, Drager R, Eibel H, Fischer B, Schaffer AA, Mages HW *et al*: Homozygous loss of ICOS is associated with adult-onset common variable immunodeficiency. Nat Immunol 2003, 4(3):261-8.
10. Salzer U, Chapel HM, Webster AD, Pan-Hammarstrom Q, Schmitt-Graeff A, Schlesier M, Peter HH, Rockstroh JK, Schneider P, Schaffer AA *et al*: Mutations in TNFRSF13B encoding TACI are associated with common variable immunodeficiency in humans. Nat Genet 2005, 37(8):820-8.
11. Warnatz K, Salzer U, Rizzi M, Fischer B, Gutenberger S, Bohm J, Kienzler AK, Pan-Hammarstrom Q, Hammarstrom L, Rakhmanov M *et al*: B-cell activating factor receptor deficiency is associated with an adult-onset antibody deficiency syndrome in humans. Proc Natl Acad Sci U S A 2009, 106(33):13945-50.
12. Lopez-Herrera G, Tampella G, Pan-Hammarstrom Q, Herholz P, Trujillo-Vargas CM, Phadwal K, Simon AK, Moutschen M, Etzioni A, Mory A *et al*: Deleterious mutations in LRBA are associated with a syndrome of immune deficiency and autoimmunity. Am J Hum Genet 2012, 90(6):986-1001.
13. Zhou Q, Lee GS, Brady J, Datta S, Katan M, Sheikh A, Martins MS, Bunney TD, Santich BH, Moir S *et al*: A hypermorphic missense mutation in PLCG2, encoding phospholipase Cgamma2, causes a dominantly inherited autoinflammatory disease with immunodeficiency. Am J Hum Genet 2012, 91(4):713-20.
14. Salzer E, Santos-Valente E, Klaver S, Ban SA, Emminger W, Prengemann NK, Garncarz W, Mullauer L, Kain R, Boztug H *et al:* B-cell deficiency and severe autoimmunity caused by deficiency of protein kinase C delta. Blood 2013, 121(16):3112-6.
15. van Zelm MC, Reisli I, van der Burg M, Castano D, van Noesel CJ, van Tol MJ, Woellner C, Grimbacher B, Patino PJ, van Dongen JJ *et al*: An antibody-deficiency syndrome due to mutations in the CD19 gene. N Engl J Med 2006, 354(18):1901-12.
16. Kuijpers TW, Bende RJ, Baars PA, Grummels A, Derks IA, Dolman KM, Beaumont T, Tedder TF, van Noesel CJ, Eldering E *et al*: CD20 deficiency in humans results in impaired T cell-independent antibody responses. J Clin Invest 2010, 120(1):214-22.

17. Thiel J, Kimmig L, Salzer U, Grudzien M, Lebrecht D, Hagena T, Draeger R, Voelxen N, Bergbreiter A, Jennings S *et al:* Genetic CD21 deficiency is associated with hypogammaglobulinemia. J Allergy Clin Immunol 2012, 129(3):801-10 e6.
18. van Zelm MC, Smet J, Adams B, Mascart F, Schandene L, Janssen F, Ferster A, Kuo CC, Levy S, van Dongen JJ *et al*: CD81 gene defect in humans disrupts CD19 complex formation and leads to antibody deficiency. J Clin Invest 2010, 120(4):1265-74.
19. Fliegauf M, Bryant VL, Frede N, Slade C, Woon ST, Lehnert K, Winzer S, Bulashevska A, Scerri T, Leung E *et al*: Haploinsufficiency of the NF-kappaB1 Subunit p50 in Common Variable Immunodeficiency. Am J Hum Genet 2015, 97(3):389-403.
20. van der Burg M, van Zelm MC, Driessen GJ, van Dongen JJ: New frontiers of primary antibody deficiencies. Cell Mol Life Sci 2012, 69(1):59-73.
21. Orange JS, Glessner JT, Resnick E, Sullivan KE, Lucas M, Ferry B, Kim CE, Hou C, Wang F, Chiavacci R *et al*: Genome-wide association identifies diverse causes of common variable immunodeficiency. J Allergy Clin Immunol 2011, 127(6):1360-7 e6.
22. Ferreira RC, Pan-Hammarstrom Q, Graham RR, Gateva V, Fontan G, Lee AT, Ortmann W, Urcelay E, Fernandez-Arquero M, Nunez C *et al*: Association of IFIH1 and other autoimmunity risk alleles with selective IgA deficiency. Nat Genet 2010, 42(9):777-80.
23. Gathmann B, Goldacker S, Klima M, Belohradsky BH, Notheis G, Ehl S, Ritterbusch H, Baumann U, Meyer-Bahlburg A, Witte T *et al*: The German national registry for primary immunodeficiencies (PID). Clin Exp Immunol 2013, 173(2):372-80.
24. Gathmann B, Grimbacher B, Beaute J, Dudoit Y, Mahlaoui N, Fischer A, Knerr V, Kindle G, Party ERW: The European internet-based patient and research database for primary immunodeficiencies: results 2006-2008. Clin Exp Immunol 2009, 157 Suppl 1:3-11.
25. Tseng CW, Lai KL, Chen DY, Lin CH, Chen HH: The Incidence and Prevalence of Common Variable Immunodeficiency Disease in Taiwan, A Population-Based Study. PLoS One 2015, 10(10):e0140473.
26. Gathmann B, Mahlaoui N, Ceredih, Gerard L, Oksenhendler E, Warnatz K, Schulze I, Kindle G, Kuijpers TW, Dutch WID *et al*: Clinical picture and treatment of 2212 patients with common variable immunodeficiency. J Allergy Clin Immunol 2014, 134(1):116-26.
27. Dantas EO, Aranda CS, Rego Silva AM, Tavares FS, Severo Ferreira JF, de Quadros Coelho MA, de Siqueira Kovalhuk LC, Roxo Junior P, Toledo EC, Porto Neto AC *et al*: Doctors' awareness concerning primary immunodeficiencies in Brazil. Allergol Immunopathol (Madr) 2015, 43(3):272-8.
28. Visitsunthorn N, Hengcrawit W, Jirapongsananuruk O, Luangwedchakam V: Immunoglobulin G (IgG) subclass deficiency in Thai children. Asian Pac J Allergy Immunol 2011, 29(4):332-7.
29. Boyle RJ, Le C, Balloch A, Tang ML: The clinical syndrome of specific antibody deficiency in children. Clin Exp Immunol 2006, 146(3):486-92.
30. Karaca NE, Karadeniz C, Aksu G, Kutukculer N: Clinical and laboratory evaluation of periodically monitored Turkish children with IgG subclass deficiencies. Asian Pac J Allergy Immunol 2009, 27(1):43-8.
31. Ruuskanen O, Nurkka A, Helminen M, Viljanen MK, Kayhty H, Kainulainen L: Specific antibody deficiency in children with recurrent respiratory infections: a controlled study with follow-up. Clin Exp Immunol 2013, 172(2):238-44.
32. Gaschignard J, Levy C, Chrabieh M, Boisson B, Bost-Bru C, Dauger S, Dubos F, Durand P, Gaudelus J, Gendrel D *et al*: Invasive pneumococcal disease in children can reveal a primary immunodeficiency. Clin Infect Dis 2014, 59(2):244-51.
33. Shima H, Kitagawa H, Wakisaka M, Furuta S, Hamano S, Aoba T: The usefulness of laryngotracheal separation in the treatment of severe motor and intellectual disabilities. Pediatr Surg Int 2010, 26(10):1041-4.
34. Kusters MA, Verstegen RH, Gemen EF, de Vries E: Intrinsic defect of the immune system in children with Down syndrome: a review. Clin Exp Immunol 2009, 156(2):189-93.
35. Davies EG: Immunodeficiency in DiGeorge Syndrome and Options for Treating Cases with Complete Athymia. Front Immunol 2013, 4:322.

36. Busse PJ, Razvi S, Cunningham-Rundles C: Efficacy of intravenous immunoglobulin in the prevention of pneumonia in patients with common variable immunodeficiency. J Allergy Clin Immunol 2002, 109(6):1001-4.
37. Favre O, Leimgruber A, Nicole A, Spertini F: Intravenous immunoglobulin replacement prevents severe and lower respiratory tract infections, but not upper respiratory tract and non-respiratory infections in common variable immune deficiency. Allergy 2005, 60(3):385-90.
38. Quinti I, Soresina A, Guerra A, Rondelli R, Spadaro G, Agostini C, Milito C, Trombetta AC, Visentini M, Martini H *et al*: Effectiveness of immunoglobulin replacement therapy on clinical outcome in patients with primary antibody deficiencies: results from a multicenter prospective cohort study. J Clin Immunol 2011, 31(3):315-22.
39. Schubert D, Bode C, Kenefeck R, Hou TZ, Wing JB, Kennedy A, Bulashevska A, Petersen BS, Schaffer AA, Gruning BA *et al*: Autosomal dominant immune dysregulation syndrome in humans with CTLA4 mutations. Nat Med 2014, 20(12):1410-6.
40. Schepp J, Bulashevska A, Mannhardt-Laakmann W, Cao H, Yang F, Seidl M, Kelly S, Hershfield M, Grimbacher B: Deficiency of Adenosine Deaminase 2 Causes Antibody Deficiency. J Clin Immunol 2016, 36(3):179-86.
41. Fischer A, Hacein-Bey Abina S, Touzot F, Cavazzana M: Gene therapy for primary immunodeficiencies. Clin Genet 2015, 88(6):507-15.
42. Ozen A, Baris S, Karakoc-Aydiner E, Ozdemir C, Bahceciler NN, Barlan IB: Outcome of hypogammaglobulinemia in children: immunoglobulin levels as predictors. Clin Immunol 2010, 137(3):374-83.
43. Janssen WJ, Nierkens S, Sanders EA, Boes M, van Montfrans JM: Antigen-specific IgA titres after 23-valent pneumococcal vaccine indicate transient antibody deficiency disease in children. Vaccine 2015, 33(46):6320-6.
44. Brignier AC, Mahlaoui N, Reimann C, Picard C, Kracker S, de Vergnes N, Rieux-Laucat F, Frange P, Suarez F, Neven B *et al*: Early-onset hypogammaglobulinemia: A survey of 44 patients. J Allergy Clin Immunol 2015, 136(4):1097-9 e2.

SUMMARY

Children often suffer from respiratory infections, which are usually innocent and self-limiting. However, in some cases these infections continuously recur; this can be a sign of underlying primary immunodeficiency (PID). Identifying children with PID, especially the milder forms, among the many children seen in everyday practice, can be challenging. This is further complicated because of the maturing immune system in children; it is often not easy to distinguish a physiologically immature from a pathologically dysfunctional immune system. Furthermore, little is known about the prevalence and prognosis of hypogammaglobulinemia in children, the most common form of primary immunodeficiency. The aim of this thesis was (1) to get more insight into the pitfalls of the developing immune system for doctors confronted by children with potential primary immunodeficiency and (2) to study in detail the clinical picture of hypogammaglobulinemia in children using data from (inter)national registries and surveys.

In *Part 1*, we describe extensive reference values for newly described lymphocyte subpopulations in children. In children it is necessary to use age-matched reference values, because of their developing immune system. The then available age-matched reference values were determined in relatively small samples, which question their accuracy especially in the youngest children. Moreover, age-matched reference values for newly discovered lymphocyte subpopulations that are thought to play an important role in the pathogenesis of primary immunodeficiency were not yet determined.
Chapter 1 shows the age-matched reference values for B cell subpopulations we determined in healthy children using leftover blood. Because healthy young children do not often need blood examination, the number of subjects in the different age groups were 10-21. Therefore, we used the statistical method of tolerance intervals which determines a reliable interval despite the small numbers tested per age group. The presented reference values define a range with a 95% chance that 90% of healthy children will have absolute numbers within the reported range. This study again showed that the greatest changes in the composition of the B cell compartment occur in the first 2 years of life. The increase in B cells in the first months of life is almost entirely caused by expansion of the naive B-lymphocyte pool, and to a small extent by expansion of transitional cells.

Immunophenotyping of B cell subpopulations is increasingly used to classify patients with common variable immunodeficiency disorders (CVID) into subgroups related to a different clinical prognosis. CVID is a heterogeneous group of primary immunodeficiency diseases characterized by late-onset hypogammaglobulinemia. We compared the obtained age-matched reference values with the EUROclass CVID classification scheme. Forty out of our 97 normal healthy children could be 'classified' in the CVID subgroup with the highest risk of complications according to the EUROclass system. Even though most of these children were younger than 2 years of age, when a diagnosis of CVID cannot yet be made, we advise to be cautious when using these classification schemes in children. We also looked at the frequency and intensity of TACI and BAFF-R expression

on B cells. Both are transmembrane receptors that belong to the TNF family and are able to transduce signals that result in isotype switching of B cells. Mutations in TACI and BAFF-R have been described in CVID patients. We demonstrated that age is the primary determinant of TACI expression on B cells (i.e. % of $TACI^+$ B cells). This means that a low number of TACI-positive B cells in young children is not automatically indicative of a potential TACI mutation. We did not find any effect of age on the BAFF-R expression (frequency and intensity) of B cells.

The same methods and comparable statistics were used to determine the reference values for T cell subpopulations in the healthy children, which we describe in *chapter 2.* Next to the different naive, effector and memory $CD4^+$ and $CD8^+$ T cells, we also looked at recent thymic emigrants, regulatory T cells and $CXCR5^+$ helper T cells using the whole lysed blood method, which is most often used in diagnostic procedures. We show that total absolute T cell numbers increase 1.4-fold during the first months of life, and that after 9–15 months they gradually decrease threefold to adult values. The increase in the first months of life is mainly caused by the expansion of recent thymic emigrants and naive cells. Helper and cytotoxic T cell subpopulations show the same pattern. Regulatory T cells increase in the first 5 months of life and then gradually decrease to adult values, although the absolute numbers remain small. $CXCR5^+$ memory T cells are virtually absent at birth and increase in numbers during the first year of life, paralleling the start of infantile IgA and IgG production. Our age-related reference values for these subpopulations can be used to help discern normal from abnormal T cell development in children.

Antigen exposure is a trigger for proliferation and differentiation of lymphocytes in the peripheral blood. During this process of differentiation antigen exposure also leads to the process of somatic hypermutation (SHM). SHM is a programmed process in which mutations in the DNA coding for the variable regions of immunoglobulin genes are induced to generate higher-affinity B cell receptors. It has not been studied in detail how SHM develops with increasing age, nor how it is related to the development of populations of different memory B cells. In *chapter 3*, we determined the SHM levels in different age groups using the Igκ-REHMA assay, an analysis that estimates the amount of SHM in expressed Vk3/20 gene segments in a specific sequence motif (hot-spot), displayed as percentage of mutated segments within the total peripheral blood cell population. We show that the estimated level of SHM increases rapidly during the first 2 years of life. This increase was associated with changes in size of the two B cell subpopulations that contain SHM (switched memory B cells [smB] and non-switched memory B cells [nsmB]), but this could not explain the total observed increased SHM frequency. We hypothesize that the increase of SHM in childhood reflects an increase of the proportion of memory B cells originating from secondary GC-dependent B cell responses as well as an increase of the SHM frequency within the memory B cell compartment, reflecting immune maturation.

In *Part 2* we study in detail the clinical picture of antibody deficiency in children focusing on hypogammaglobulinemia using data from (inter)national registries and surveys.

Chapter 4 shows the results from the PedPAD study: a study using the ESID online database. Characteristics from 2076 children with hypogammaglobulinemia collected in 46 centres situated in 18 different countries are described. Unfortunately, the data set showed several limitations, making interpretation of the data challenging. There were obvious data entry errors and many missing values. The data showed there were country-related differences in the use of diagnostic criteria for the classification of different types of primary hypogammaglobulinemia. Analysis of the data was performed on the cleaned data set of 'total hypogammaglobulinemia patients'. The most striking observation was the predominance of male patients in this group of children with primary hypogammaglobulinemia (n = 1292, 63%). This male predominance was observed in each of the 18 countries involved and had not been described in the literature before. Moreover, the boys were younger at diagnosis (mean age males 5.3 years; mean age females 5.8 years) and complications, like auto-immunity, polyclonal lymphocytic infiltration, enteropathy and lymphoid malignancy, were more frequently reported in boys (12%) compared to girls (5%). This male predominance suggests that patients with an undetected or unknown X-linked genetic disease are included in this group of children registered as primary hypogammaglobulinemia.

In *chapter 5,* we describe a cohort of 49 children with IgG-subclass deficiency and/or specific polysaccharide antibody disorders (SPAD) collected from secondary and tertiary centers all over the Netherlands using *the national surveillance institute for pediatric diseases* ("Nederlands Signalerings Centrum voor Kindergeneeskunde" [NSCK]) to collect the data. Both diseases are generally considered as milder forms of hypogammaglobulinemia. IgG-subclass deficiency is defined as a deficiency in one or more IgG-subclasses (>2 SD below age-matched reference values) with normal or near normal IgG concentration. SPAD is diagnosed when there is profound alteration of the antibody response to polysaccharide antigens. This was the first study published in a Western-European cohort, and the first that included children followed outside tertiary centers. The most prevalent IgG-subclass deficiency in our cohort was IgG2 deficiency. This is in contrast with other published cohorts, where IgG3 deficiency was most common. These cohorts contained a higher number of children on prophylactic antibiotics, and were performed in tertiary populations. This may explain this difference in phenotype. However, ten percent of the children in our study with 'milder' immunodeficiencies already showed bronchiectasis. Again, significantly more boys (67%) than girls were reported. After the age of 9, only boys were reported, and only boys (11/33 boys at baseline) showed progressive immunodeficiency during follow-up. Overall, twenty-five percent of the children with IgG-subclass deficiency and/or SPAD recovered during follow-up. The majority of children (69%) were reported by four secondary hospitals with a pediatric immunologist in the staff, which raises the question whether many of these

patients are missed. If we want to identify more patients, probably the awareness of these diseases among general pediatricians should increase.

A special group of children with recurrent infections are children with chromosomal disorders. In *chapter 6*, we present a cohort of children with chromosomal aberration and primary immunodeficiency recruited with a survey among ESID members. We invited all members of the ESID to report patients with chromosomal aberration in combination with one or more identified immunological abnormalities relating to primary immunodeficiency. Our call identified 46 patients with a wide variety of chromosomal aberration associated with immunological abnormalities potentially relating to primary immunodeficiency. This is the largest cohort reported in the literature so far (42 isolated cases, and twice 2 patients from separate families). Their most common clinical presentation was 'recurrent ENT and airway infections', which triggered their physician to perform immunological investigations. Not surprisingly, the detected primary immunodeficiencies were mostly 'predominantly antibody deficiencies', ranging from IgG-subclass deficiency and/or SPAD to severe hypogammaglobulinemia or even agammaglobulinemia in one patient. Recognition of hypogammaglobulinemia in these patients, and not automatically ascribing the recurrent infections to factors such as anatomic and/or neuromuscular problems, is important because these infections can be prevented with interventions like antibiotic prophylaxis or immunoglobulin substitution.

The studies in this thesis demonstrate the challenge of diagnosing predominantly antibody disorders in children. This is partly due to their maturing immune system, but also due to the unawareness of doctors. Our developed age-matched reference values provide some grip to distinguish normal from disease. As shown, analysis of large patient cohorts using registries and/or surveys can detect unknown patterns, like the predominance of boys with hypogammaglobulinemia, which gives entries for future research.

NEDERLANDSE SAMENVATTING VOOR NIET INGEWIJDEN

Jonge kinderen hebben vaak last van infecties. Deze zijn meestal onschuldig en gaan vanzelf weer over. In sommige gevallen komen de infecties steeds terug omdat de afweer niet goed is. De meest voorkomende afweerstoornis bij kinderen is een tekort aan antistoffen ('immuunglobulinen') in het bloed. Dit wordt 'hypogammaglobulinemie' genoemd. Er is nog maar weinig bekend over hypogammaglobulinemie bij kinderen. Hoeveel kinderen hebben hier eigenlijk last van? Wat zijn hun klachten? En hoe is het beloop van de ziekte? Daarnaast is het vaak lastig om een afweerstoornis bij kinderen vast te stellen. Dit heeft te maken met het feit dat het immuunsysteem nog in ontwikkeling is. Bij de geboorte zijn kinderen nog maar weinig beschermd tegen infecties, ze worden deels beschermd door de antistoffen die ze via de placenta van hun moeder hebben gekregen. In de loop van het eerste levensjaar komt de eigen antistofproductie op gang. Echter, net zoals het ene kind vroeg kan lopen en praten en dit bij een ander kind langer duurt maar uiteindelijk ook lukt, is er een grote variatie in de snelheid van uitrijping van het immuunsysteem. Het is vaak lastig bij (jonge) kinderen 'trage rijping' van 'gestoorde afweer' te onderscheiden. Het doel van dit proefschrift was om meer inzicht te krijgen in het zich ontwikkelende immuunsysteem bij kinderen en om de kenmerken van kinderen met een hypogammaglobulinemie beter in kaart te brengen. Hiervoor is gebruik gemaakt van (internationale) patiënten registratie systemen en enquêtes onder collega's om patiënten met deze ziektebeelden te verzamelen.

In *hoofdstuk 1 en 2* laten we de normaalwaarden zien van verschillende soorten afweercellen bij gezonde kinderen. Het immuunsysteem bestaat uit veel soorten afweercellen die allemaal hun eigen functie hebben. Wij hebben naar de lymfocyten gekeken. Dit zijn witte bloedcellen die een specifieke afweerreactie op bacteriën en virussen kunnen maken. Daarnaast zorgen ze er ook voor dat er geheugen wordt gevormd, zodat je een volgende keer niet, of minder, ziek wordt van eenzelfde verwekker. Lymfocyten bestaan voor een belangrijk deel uit B cellen en T cellen. De B cellen kunnen zich ontwikkelen tot antistof producerende plasmacellen. De antistoffen die deze plasmacellen maken zijn specifiek gericht tegen een bepaalde ziekteverwekker. Ze markeren de ziekteverwekker zodat andere afweercellen in het lichaam deze beter kunnen herkennen en opruimen. Bepaalde soorten T cellen (de cytotoxische T cellen) kunnen bijvoorbeeld zo de ziekteverwekkers uit de weg ruimen. Andere T cellen (de helper T cellen) helpen de B cellen om nog sterkere en specifiekere antistoffen te maken zodra ze een ziekteverwekker voor de eerste keer zijn tegengekomen. We zien in onze studies dat er vooral in de eerste twee levensjaren grote veranderingen in de aantallen van de verschillende B en T celsoorten plaatsvinden. Vanaf de leeftijd van twee jaar zijn er minder schommelingen in de aantallen te zien, maar duurt het nog een aantal jaar voordat de volwassen waarden zijn bereikt. Omdat de aantallen van de verschillende lymfocyten bij kinderen zo duidelijk verschillen met die van volwassenen, dient men voorzichtig te zijn met het toepassen van classificatie criteria die op basis van lymfocyten subpopulatie aantallen zijn opgesteld voor volwassen patiënten met afweerstoornissen. De door ons gevonden normaalwaarden kunnen in de dagelijkse praktijk gebruikt worden

om onderscheid te maken tussen een zich normaal ontwikkelend en een afwijkend immuunsysteem bij kinderen.

In *hoofdstuk 3* gaan we meer op de details van de antistofproductie van B cellen in. Zoals gezegd helpen de T helper cellen de B cellen om nog sterkere en specifiekere antistoffen te maken. Dit gebeurt in de zogenaamde 'germinal centers' in de lymfeklieren. Het DNA (erfelijke materiaal) van de B cellen dat codeert voor de antistoffen wordt een klein beetje veranderd met als doel om een sterkere binding aan ziekteverwekkers te krijgen. Dit proces wordt somatische hypermutatie (SHM) genoemd. We hebben in dezelfde gezonde kinderen gekeken naar de hoeveelheid van deze DNA veranderingen in verschillende leeftijdsgroepen. Ook hier zagen we dat er in de eerste twee levensjaren de grootste veranderingen optraden. Vanaf twee jaar blijft de hoeveelheid van deze DNA veranderingen nagenoeg stabiel. De toename van deze DNA veranderingen in de eerste twee levensjaren komt enerzijds door de toename van het aantal B cellen waarin deze SHM kan plaatsvinden, maar kan niet alleen hierdoor verklaard worden. Wij denken dat ook een toename van frequentie van de SHM per cel hiervoor verantwoordelijk is.

Het volgende deel van het proefschrift bekijkt de kenmerken van kinderen met een hypogammaglobulinemie. Als eerste beschrijven we in *hoofdstuk 4* een groep van 2076 kinderen met hypogammaglobulinemie in de PedPAD studie. De gegevens van deze kinderen hebben we verzameld door gebruik te maken van een reeds bestaande Europese patiënten registratie die opgezet is door de European Society for Immunodeficiencies (ESID online database). De kinderen waren afkomstig van 46 ziekenhuizen uit 18 verschillende landen. Doordat er in de verschillende landen andere diagnostische criteria werden gebruikt, was het lastig om verschillende groepen met elkaar te vergelijken. We hebben daarom alle kinderen met een hypogammaglobulinemie bij elkaar genomen. Het meest opvallende was de grote hoeveelheid aan jongens in dit cohort (n = 1292, 63%). Dit zagen we terug in alle landen en was niet eerder uit andere studies gerapporteerd. Ook zagen we dat de jongens gemiddeld jonger waren bij diagnose (gemiddelde leeftijd jongens 5,3 jaar; gemiddelde leeftijd meisjes 5,8 jaar) en dat ze daarnaast ook vaker last hadden van complicaties (12% van de jongens tegen 5% van de meisjes).

In *hoofdstuk 5* bekijken we een groep van Nederlandse kinderen met een milde vorm van hypogammaglobulinemie. Deze groep kinderen hebben we verzameld via het Nederlands Signalerings Centrum voor Kindergeneeskunde (NSCK) waar in periode van 1 maart 2009 tot 1 november 2011 kinderen met deze milde vorm van hypogammaglobulinemie werden verzameld. Dit gaat om kinderen met een tekort in 1 of meerdere antistof subtypes (IgG-subklasse deficiëntie). De totale hoeveelheid antistoffen in het bloed is dan wel (nog) normaal. Ook kinderen die geen specifieke antistoffen tegen suikerkapsels van bacteriën konden maken werden bekeken (specifieke polysacharide antistof deficiëntie [SPAD]). Ondanks het feit dat deze afweerstoornissen beschouwd

worden als 'mildere' ziektes, hadden 10% van de kinderen al chronische schade aan de longen opgelopen. Daarnaast zagen we ook hier dat de meerderheid van de kinderen jongens waren (67%). Sterker nog, vanaf de leeftijd van 9 jaar bleven er alleen nog maar jongens over in de groep. Ook waren het alleen jongens die tijdens de follow-up een verslechtering van de ziekte lieten zien. Vijfentwintig procent van de kinderen liet uiteindelijk een normalisatie van de antistoffen zien tijdens follow-up. Opvallend was dat kinderen met een IgG-subklasse deficiëntie en/of SPAD vooral gerapporteerd werden door kinderarts-immunologen en minder vaak door een algemeen kinderarts. Wij denken dat als er meer aandacht komt voor het opsporen van onderliggende afweerstoornissen door algemene kinderartsen, er in de toekomst wellicht meer kinderen met deze ziekte aan het licht kunnen komen.

Ten slotte beschrijven we in *hoofdstuk 6* een speciale groep kinderen met recidiverende infecties, namelijk kinderen met een afwijking in de chromosomen (de dragers van het erfelijk materiaal). We weten dat deze kinderen vaker last hebben van infecties, maar dit wordt vaak geweten aan andere oorzaken zoals het feit dat ze minder sterk zijn, of zich vaak verslikken. Natuurlijk spelen deze factoren mee, maar het blijkt belangrijk om ook bij deze kinderen de afweer goed te bekijken. Een oproep via de ESID leverde 46 patiënten op die zowel een afwijking in de chromosomen hadden als een afweerstoornis. De meeste patiënten hadden last van recidiverende KNO en luchtweginfecties. De meest voorkomende afweerstoornis in deze groep bleek hypogammaglobulinemie te zijn.

Samenvattend laat dit proefschrift de uitdagingen zien van het vaststellen van afweerstoornissen bij kinderen. Dit heeft enerzijds te maken met het zich nog ontwikkelende immuunsysteem maar ook de onbekendheid bij artsen speelt hierin een rol. De door ons opgestelde normaalwaarden van lymfocytensubpopulaties kunnen helpen bij het vaststellen bij welk kind met frequente infecties mogelijk een afweerstoornis aanwezig is. Daarnaast hebben wij laten zien dat meer jongens last hebben van hypogammaglobulinemie dan meisjes. Toekomstig onderzoek is nodig om uit te vinden wat hier de oorzaak van is. Daarnaast is het belangrijk om de bekendheid met hypogammaglobulinemie bij artsen te vergroten zodat meer kinderen met een afweerstoornis herkend worden en een passende behandeling kunnen krijgen.

ADDENDUM

Dankwoord
Curriculum Vitae
Publications
Abbreviations

Dankwoord

Dit proefschrift is niet alleen tot stand gekomen dankzij mijn inspanningen, maar ook door de inzet en steun van vele andere enthousiaste en bereidwillige mensen. Zonder jullie was dit mij nooit gelukt, dankjewel!

Allereerst wil ik alle kinderen (en ouders) die hebben deelgenomen aan de verschillende onderzoeken bedanken. Zonder jullie bereidheid tot deelname was dit onderzoek überhaupt niet mogelijk geweest.

Daarnaast wil ik graag mijn promotoren, co-promotor en andere leden van de promotiecommissie bedanken.

Prof. dr. E. de Vries, beste Esther, zonder jou was dit proefschrift er zeker nooit gekomen. Los van het feit dat ik in een rijdende trein kon springen, heb je mij ook tussentijds gestimuleerd om door te gaan en samen met mij projecten gezocht die ik naast mijn reguliere werk kon blijven uitvoeren. Dat het een meerjarig traject ging worden, was voor jou geen belemmering. En door jouw immer snelle respons op al mijn e-mails, kon ik vaak in korte tijd heel veel werk verzetten. Enorm bedankt voor je begeleiding de afgelopen jaren.

Prof. dr. N. Wulffraat, beste Nico, wat fijn dat jij mijn promotor wilt zijn. Jouw kritische vragen en kijk op het grotere geheel zijn van grote waarde geweest voor mijn uiteindelijke proefschrift. Hartelijk dank voor de altijd prettige samenwerking, fijn dat jouw deur altijd open staat voor mij.

Dr. M. de Flier, beste Michiel, jouw bijdrage aan het laatste deel van mijn proefschrift was super. Jij hebt de gave om complexe zaken op een heel heldere manier te verwoorden en uit te leggen. Dit heeft mij vooral bij het schrijven van de discussie enorm geholpen. Dankjewel hiervoor. Ik hoop dat we in de toekomst nog vaker kunnen samenwerken.

Prof. dr. D.H. Bakker, prof. dr. P.M. van Hagen, prof. dr. F.H.J. van den Hoogen, dr. J.G. Noordzij en prof. dr. G.T. Rijkers wil ik graag bedanken voor het bestuderen van dit manuscript. Prof. dr. F.H.J. van den Hoogen, beste Frank, aan jouw een speciaal woord van dank voor je vertrouwen in mij als (hopelijk) toekomstig kinderreumatoloog in het Nijmeegse. Ik kijk er naar uit om weer 'terug' te komen en vind het fijn dat jij deel uit wilt maken van de promotiecommissie.

Ook wil ik alle co-auteurs van alle artikelen heel hartelijk bedanken voor hun hulp en commentaren.

Extra dank aan Eugenie Gemen, zonder jou Eugenie was het normaalwaarden onderzoek niet (zo snel) gegaan. Naast het feit dat jij alle analyses in het lab hebt uitgevoerd (of hebt laten uitvoeren), was jij tijdens mijn afwezigheid de coördinator van dit onderzoek in het Jeroen Bosch Ziekenhuis. Ik kon blindelings op jou vertrouwen, dankjewel.

Beste Roeland van Hout, hartelijk dank voor al je hulp bij de statische analyses. Zonder jou was dit deel een ware uitdaging geweest, nu ging het fluitend, dankjewel!

Gertjan Driessen en Mirjam van der Burg, ook aan jullie een speciaal woord van dank voor de samenwerking in de referentiewaarden studie en het onderzoek naar somatische hypermutatie. Ik ben dankbaar dat ik gebruik heb kunnen maken van jullie kennis en faciliteiten op het lab.

Dear Benjamin Gathmann, a special thanks to you for your cooperation in the PedPAD study and your help with analysing the data from the ESID Online Database. You are the fastest email responder I have ever met.

Beste Yumely, dankjewel voor het verzamelen van de patiëntengegevens in de diverse ziekenhuizen voor de IgG-subclasse deficiëntie en/of SPAD studie.

Natuurlijk wil ik ook alle collega's van de afgelopen jaren bedanken voor de prettige samenwerking. Alle arts-assistenten in het Jeroen Bosch Ziekenhuis en het Radboudumc tijdens mijn opleidingstijd, het waren gezellige tijden!

Beste Ruud en Maaike, hoewel jullie promotie al weer even geleden is, waren we in het begin toch een soort 'onderzoeksgroep'. Dankjewel voor het beantwoorden van al mijn (praktische) vragen.

Beste onderzoekscollega's bij Tranzo, dankjewel voor het veilige en inspirerende onderzoeksklimaat waar ik nu af en toe van mag mee genieten als externe promovendus.

Extra dank aan de kinderartsen in het Jeroen Bosch Ziekenhuis voor het vertrouwen in mij, al vanaf het vroege begin van mijn loopbaan, en de mogelijkheid om nu ook als kinderarts mijn steentje te kunnen bijdragen. Speciale dank aan dr. Hans Hoekstra, zonder jou had ik nooit zo'n vliegende start in de kindergeneeskunde kunnen maken, ik blijf je daar altijd dankbaar voor.

Dr. Jos Draaisma, dankjewel voor je prettige coachende opleidersrol. Ik heb veel van je geleerd.

Alle reumatologen in de St. Maartenskliniek, dankjewel voor mijn zeer leerzame stage bij de 'volwassen' reumatologie, ik kijk er naar uit om in de toekomst weer met jullie samen te werken.

De kinderreumatologen en -immunologen in het Wilhelmina Kinderziekenhuis, beste Annet, Bas, Berend, Caroline, Jaap-Jan, Joost & Joost, en Joris, wat een voorrecht dat ik bij jullie een deel van mijn opleiding tot kinderreumatoloog heb mogen volgen. Een betere en fijnere leerschool had ik me niet kunnen wensen, ik voel me heel welkom bij jullie.

En last, but certainly not least, beste Esther Hoppenreijs, onze samenwerking gaat nu pas echt beginnen en daar ben ik echt heel blij mee. Dankjewel voor de mogelijkheden die jij voor mij gecreëerd hebt, ik hoop maar dat ik alle verwachtingen waar kan maken.

Zeker niet minder belangrijk een dankwoord aan al mijn lieve vrienden en (schoon)familie. Alleen al het feit dat jullie er zijn is genoeg!

Mijn lieve paranymfen, Annelieke en Susan, wat vind ik het fijn dat jullie mij willen ondersteunen bij mijn promotie. Op jullie vriendschap kan ik altijd rekenen, dat vind ik heel bijzonder, dankjewel.

Mijn lieve BoegBeeldig vriendinnetjes, Annedien, Bettine, Hanneke, Marije, Nieke, Steffie, Tine en Tirza, dankjewel voor al jullie gezelligheid de afgelopen jaren. Ik kijk altijd enorm uit naar onze momenten samen, never a dull moment. Jullie zijn allemaal precies goed zoals jullie zijn.

Lieve Nicky, ik ben blij dat wij elkaar tijdens de studie zijn tegengekomen. Jouw oprechte interesse, enthousiasme en positivisme maken mij blij.

Lieve Esmee en Iris, wat fijn dat ik het met jullie over alledaagse dingen kan hebben, heerlijk.

Lieve Henny en Nel, bedankt dat wij altijd op jullie kunnen rekenen.

Leef oma, wat fijn dat geej heej nog beej kint zien.

Leef pap, mam en Tom, door óg bin ich gewaore wae ich bin. Dankjewel veur n fijne kingertied en veur nog idderskier alle fijne momenten samen.

Mijn lieve Sophie en Julius, dat ik jullie mama mag zijn, is werkelijk waar het allermooiste in mijn leven. Ik zal er altijd voor jullie zijn.

Allerliefste Raymond, als jouw ogen glinsteren, dan doen die van mij dat ook. Met jou ben ik gelukkig!

Curriculum vitae

Ellen Johanna Hubertina Schatorjé werd geboren op 27 december 1983 in Venlo. Ze groeide op in Tegelen samen met haar ouders en broertje. Na het behalen van haar VWO diploma (cum laude) is zij in 2001 gestart met de studie geneeskunde in Maastricht. Tijdens de geneeskunde opleiding heeft zij zich tevens verdiept in het Nederlands Gezondheidsrecht.

In 2007 behaalde zij haar artsexamen (cum laude) en begon zij met haar baan als arts-assistent kindergeneeskunde in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch. In 2008 kon zij met haar specialisatie tot kinderarts beginnen. Haar perifere opleiding heeft ze gevolgd in het Jeroen Bosch Ziekenhuis te 's-Hertogenbosch onder leiding van dr. H. Hoekstra en vervolgens prof. dr. E. de Vries. Het academische deel van de opleiding tot kinderarts vond plaats in het Radboudumc onder leiding van dr. J. Draaisma. Tijdens deze opleiding is zij ook gestart met onderzoek naar het afweersysteem bij kinderen wat uiteindelijk geleid heeft tot dit proefschrift.

Na het afronden van haar specialisatie tot kinderarts is zij begonnen met een fellowship kinderreumatologie onder leiding van prof. dr. N. Wulffraat in het Wilhelmina Kinderziekenhuis in Utrecht en van Mw. E. Hoppenreijs in de St. Maartenskliniek en Radboudumc in Nijmegen. Dit fellowship combineert ze met het werk als algemeen kinderarts in het Jeroen Bosch Ziekenhuis in 's-Hertogenbosch. Ze verwacht dit fellowship in maart 2018 af te ronden.

Ellen woont samen met Raymond, hun dochter Sophie (2013) en zoon Julius (2016) in Rosmalen.

Publications

Primary immunodeficiency associated with chromosomal aberration – an ESID Survey.
Schatorjé EJ, van der Flier M, Seppänen M, Browning M, Morsheimer M, Henriet S, Neves JF, Vinh DC, Alsina L, Grumach A, Soler-Palacin P, Boyce T, Celmeli F, Goudouris E, Hayman G, Herriot R, Förster-Waldl E, Seidel M, Simons A, de Vries E.
Orphanet J Rare Dis. 2016 Aug 2;11(1):110.

The Challenge of Immunoglobulin-G Subclass Deficiency and Specific Polysaccharide Antibody Deficiency - a Dutch Pediatric Cohort Study.
Schatorjé EJ, de Jong E, van Hout RW, García Vivas Y, de Vries E.
J Clin Immunol. 2016 Feb;36(2):141-8.

Levels of somatic hypermutations in B cell receptors increase during childhood.
Schatorjé EJ, Driessen GJ, van Hout RW, van der Burg M, de Vries E.
Clin Exp Immunol. 2014 Nov;178(2):394-8.

The PedPAD study: boys predominate in the hypogammaglobulinaemia registry of the ESID online database.
Schatorjé EJ, Gathmann B, van Hout RW, de Vries E; PedPAD consortium.
Clin Exp Immunol. 2014 Jun;176(3):387-93.

Borrelial lymphocytoma.
Schatorjé EJ, van der Steeg H, Stelma F, Hebeda K, Warris A.
Ned Tijdschr Geneeskd. 2013;157(52):A6605.

Paediatric reference values for the peripheral T cell compartment.
Schatorjé EJ, Gemen EF, Driessen GJ, Leuvenink J, van Hout RW, de Vries E.
Scand J Immunol. 2012 Apr;75(4):436-44.

A girl with autoimmune cytopenias, nonmalignant lymphadenopathy, and recurrent infections.
Mattheij MA, **Schatorjé EJ**, Gemen EF, van de Corput L, Nooijen PT, van der Burg M, de Vries E.
Case Reports Immunol. 2012;2012:196417.

Age-matched reference values for B-lymphocyte subpopulations and CVID classifications in children.
Schatorjé EJ, Gemen EF, Driessen GJ, Leuvenink J, van Hout RW, van der Burg M, de Vries E.
Scand J Immunol. 2011 Nov;74(5):502-10.

B-lymphocyte reconstitution after repeated rituximab treatment in a child with steroid-dependent autoimmune hemolytic anemia.
van der Linde AA, **Schatorjé EJ**, van der Weij AM, Gemen EF, de Vries E.
Pediatr Rep. 2011 Sep 30;3(4):e28.

Transient hypertransaminasemia in paediatric patients with Crohn disease undergoing initial treatment with enteral nutrition.
Schatorjé EJ, Hoekstra H.
J Pediatr Gastroenterol Nutr. 2010 Sep;51(3):336-40.

Prevention and treatment of congenital syphilis.
Schatorjé EJ, Bruijn M, op de Coul ME, Busari JO.
Ned Tijdschr Geneeskd. 2007 Oct 13;151(41):2241-7.

Abbreviations

ADA2	adenosine deaminase 2
AID	activation-induced cytidine deaminase
APC	antigen presenting cell or allophycocyanin
APRIL	a proliferation-inducing ligand
BAFF-R	B cell activation factor receptor
BCR	B cell receptor
BD	Becton Dickinson
BLNK	B cell linker
bp	base pair
BTK	Bruton's kinase
CAML	calcium-modulating cyclophilin ligand
CD	cluster of differentiation
CDR	complementary determining region
CSR	class-switch recombination
CTLA-4	cytotoxic T-lymphocyte-associated 4
CVID	common variable immunodeficiency disorders
CXCR5	CXC chemokine receptor 5
EDTA	ethylenediaminetetraacetic-acid
ENT	ear-nose-throat
ESID	European Society for Immunodeficiencies
Fab	variable region of immunoglobulin
Fc	constant domain of immunoglobulin
FITC	fluoresceïne-isothiocyanate
GC	germinal center
GWAS	genome wide association study
ICOS	inducible T cell costimulator
Ig	immunoglobulin
IgH	immunoglobulin heavy chain
IGHM	μ-heavy chain
IgK-REHMA	IgK-restriction enzyme hot-spot mutation assay
IFN	interferon
IL	interleukin
IPH	idiopathic primary hypogammaglobulinemia
ISCN	international system for human cytogenetic nomenclature
ITAM	immunoreceptor tyrosine-based activation motif
IUIS	international union of immunological societies
KRECs	kappa-deleting recombination excision circles
LRBA	lipopolysaccharide responsive beige-like anchor protein
MAb	monoclonal antibody
MHC	major histocompatibility complex

NFKBIA	nuclear factor of kappa light chain gene enhancer in B cells inhibitor alpha
NK	natural killer
NSCK	Nederlands Signalerings Centrum voor Kindergeneeskunde
nsmB	non-switched memory B cells
PAD	predominantly antibody deficiencies
PD-1	programmed cell death 1
PerCP-Cy-5.5	peridinin chlorophyll protein–cyanin
PE	phycoerythrin
PedPAD	pediatric predominantly antibody deficiencies
PI3K	phosphoinositide 3-kinase
PID	primary immunodeficiencies
PLCG2	phospholipase Cγ2
PRKCD	protein C kinase δ
RT-PCR	reverse transcription–polymerase chain reaction
SCID	severe combined immunodeficiency
SHM	somatic hypermutation
smB	switched memory B cell
SNP	single nucleotide polymorphism
SPAD	specific polysaccharide antibody deficiency
TACI	transmembrane activator and calcium-modulator and cyclophilin ligand interactor
TCR	T cell receptor
Tfh	follicular T helper cell
Th	T helper cell
THI	transient hypogammaglobulinemia of infancy
TI	T cell independent
TNF	tumor necrosis factor
TRECs	T cell receptor excision circles
Treg	regulatory T cells
VDJ	variable, diversity, joining
WASP	Wiskott–Aldrich syndrome
XLA	X-linked agammaglobulinaemia
XLH-GHD	X-linked hypogammaglobulinemia and isolated growth hormone deficiency